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NUTRIÇÃO

Gut health improvement in fish and shrimp through fortified diets: focus on inflammatory condition, immune status, and microbiota diversity

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2022

U.PORTO

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D.ICBAS **2022**

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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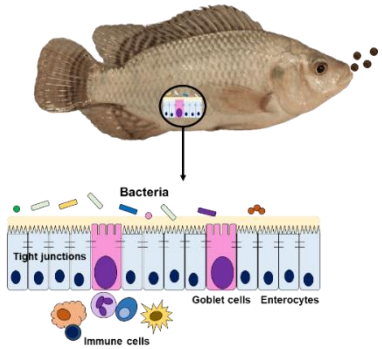
Thesis for applying to a Doctor degree in Animal Science, Specialization in Nutrition, submitted to the Institute of Biomedical Sciences Abel Salazar of the University of Porto.



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This PhD Thesis includes one scientific paper published in an international peer-review journal originating from part of the results obtained in the experimental work:

Teixeira C., Peixoto D., Hinzmann M., Santos P., Ferreira I., Pereira G.V., Dias J. and Costas B. (2022). Dietary strategies to modulate the health condition and immune responses in gilthead seabream (*Sparus aurata*) juveniles following intestinal inflammation. **Accepted in *Animals*.**

This PhD Thesis also includes three manuscripts submitted, originating from part of the experimental work:

Teixeira C., Serra C.R., Hinzmann M., Costa P.M., Dias J., Rema P. & Costas B. (2022). Nile tilapia (*Oreochromis niloticus*) health modulation with dietary oxytetracycline treatment (direct effects and dietary recovery). Submitted to ***Aquaculture***.

Teixeira C., Costa P.M., Dias J., Rema P., Costas B. & Serra C.R. (2022). Modulation of intestinal morphology and microbiota diversity by the dietary administration of oxytetracycline in Nile tilapia (*Oreochromis niloticus*). Submitted to ***Frontiers in Microbiology***.

Teixeira C., Serra C.R., Fernández-Boo S., Costa P.M., Dias J., Rema P. & Costas B. (2022). Effect of krill meal and a triglyceride-rich krill oil on the immunity and intestinal microbiota of the Pacific white shrimp (*Penaeus vannamei*) fed a high-soybean meal diet. Submitted to ***Animal***.

Acknowledgements

This work was supported by Project **FICA** (references ALG-01-0247-FEDER-047175, UIDB/04423/2020 and UIDP/04423/2020), financed by Portugal and the European Union through FEDER, COMPETE 2020, and CRESC Algarve 2020, in the framework of PORTUGAL2020; and Project **ATLANTIDA** (ref. NORTE-01-0145-FEDER-000040), supported by the North Portugal Regional Operational Program (NORTE2020), under the PORTUGAL2020 Partnership Agreement and through the European Regional Development Fund (ERDF). C. Teixeira were supported by **FCT** (Foundation for Science and Technology), Portugal and by **SPAROS Lda.** through grant PD/BDE/135541/2018.

This work was also supported by the **SANFEED** Doctoral Program (Sustainable Animal Nutrition and Feeding), led by Professor Mira da Fonseca and Professor Luísa Valente, an ANIMAL SCIENCE Programme in an industrial setting funded by FCT and coordinated by the Instituto de Ciências Biomédicas Abel Salazar from the University of Porto.

Many people contributed to this thesis. Here, I would like to deeply acknowledge and give my sincere appreciation for their invaluable contribution.

To my supervisor, **Benjamín Costas**. I am very grateful for this opportunity, for growing with you all these years, for being part of this big Animal Health and Aquaculture family! and for being able to learn a little bit more every day. Thanks for always pushing me forward and for always saying “try”. Above all, thanks for being as you are, always supportive, encouraging and guiding when everything seemed wrong. Your support and guidance were crucial.

To my co-supervisor, **Cláudia Serra**. Thanks for all your guidance and understanding, for always helping me to deepen my knowledge and for being available (just a phone call away!). Thanks for your honest and crucial support.

To my co-supervisor, Professor **Paulo Costa**. Thanks for allowing me to do “my microbiology”, for all the explanations, and for always being there.

To my co-supervisor, **Jorge Dias**. I am very grateful for this opportunity. Thanks for trying to find a little moment in your filled schedule, for making my trials possible, and for making me think “outside of the box”. Thanks for allowing me to learn every single day, to participate in many different trials, and to expand my knowledge. Your guidance was crucial.

To my **host institutions**, Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR), Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto (ICBAS-

UP), Sparos Lda., and Universidade de Trás-os-Montes e Alto Douro (UTAD) and all the people involved.

To Professor **Paulo Rema** and all his work team, for kindly providing the animal facilities, for all the guidance and for always being so helpful and friendly.

To **José Couto**. Without you, the “extreme samplings” would not be possible. Thanks for helping me with my trials, and for making them possible. Thanks for making the Sparos trials and samplings more organized!!!

To **Sparos team**. I learn a lot from you all. It was a pleasure to be a part of your team! Gabriella, thanks for all the help.

To Professor **Irina Amorim** and her work team, **Fátima and Alexandra**, for kindly providing the veterinary pathology laboratory facilities, for all the processing hours, for all the guidance.

To **Dona Elisabete** and **Joana**, from the food technology and safety laboratory, for all the help to endure the petri counts and the kind explanations.

To **Nutrimu group**, for welcoming me so friendly and for always be available to help. Rafaela your help was essential.

To all my **lab partners** (in alphabetical order! do not argue!), Ana Garcia, Ana Margarida, Ana Rocha, André, Bruno, Carlos, Cristina, Diogo, Felipe, Francisco, Inês Carvalho, Inês Ferreira, Ivan, João, Lourenço, Mariana, Marina, Paulo, Rita, Rute, Sergio, Simão, Susana, Tiago and Tonka (hope I didn´t forget anyone!). Without you, this PhD would not be the same! Thanks for all the teaching and all the laughs, for all the funny sampling trips and its quick preparation (for yesterday!), for making me stop to work and have some drinks (?!), for making my weekends funnier and playing some volley (and gain some muscle!). Mariana, your support was crucial.

To **Carla, Filipa and Juliana**, my oldest friends. Your friendship and support were crucial and unquestionable.

To **my dogs** (Baunilha and Caramelo). Their companionship and craziness always made me end up with a smile at the end of the day.

And finally, to **my parents**, my greatest support. Thanks for your patience and understanding. Your unconditional support and love were essential throughout this PhD.

Summary

Aquaculture has been the agro-industrial activity with the highest growing rate worldwide, however, disease outbreaks and escalated prices of feed ingredients are an obstacle to aquaculture's continuous growth and sustainability. On the one hand, the use of antibiotics to control pathogens and infectious diseases which may trigger many environmental and health concerns; on the other hand, the use of unconventional ingredients to replace fishmeal and fish oil which may hamper fish and shrimp health.

Nutrition plays a key role in aquaculture species growth and health, being the intestine the first target organ. Intestine is a very complex organ not only responsible for nutrient digestion and absorption, but also for the modulation of the immune system. Therefore, it is of extreme importance to understand its biology and physiology, as intestinal health might influence the overall performance and health of fish and shrimp. Moreover, since the intestine homeostasis is very prone to manipulation through diet, alternative dietary formulations can be used to control and improve intestinal health and contribute to healthier fish that are more resistant to pathogens and external factors, but also, to contribute to decreasing the inflammatory conditions originated by the use of alternative feed ingredients.

There is a lack of studies related to the mechanisms associated with the intestinal inflammatory process in both fish and shrimp species, due to the lack of standardized and robust models that allow its study. Therefore, it is of extreme importance to establish low and moderate reversible inflammatory models that allow not only the study of the intestinal inflammatory mechanisms but also enable the evaluation of the ability of feed ingredients to minimize or revert inflammatory processes and its systemic consequences. Therefore, this PhD thesis intended to study the effects of antibiotics on intestinal and animal health; to understand the impact of distant protein sources in the recovery of antibiotic therapeutics; to develop an intestinal inflammatory model to study intestinal inflammation; and to evaluate the effect of dietary health-promoting feed additives and ingredients as prophylactic tools.

In **Chapters II**, **III**, and **IV** the harmful effects of chemicals on the intestinal and animals' health were evaluated, and, although the antibiotic (oxytetracycline) used in **Chapters II** and **III** and the chemical (DSS) used in **Chapter IV** produced only a mild intestinal inflammatory process, it was possible to verify the modifications produced in the gene expression profile, oxidative stress response and dynamics of the intestinal microbiota. Therefore, these types of models are of extreme importance to understand the intestinal inflammatory process and its consequences.

Also, it was possible to verify that the health consequences of the use of antibiotics (**Chapters II** and **III**) can be reversed to some extent with different dietary formulations, however, in the case of extreme dietary formulations it can compromise growth. On the

other hand, it was also possible to use alternative dietary formulations (with feed additives in the case of **Chapter IV**) to minimize the health effects of intestinal inflammatory inducers as was the chemical DSS.

On the other hand, replacing fishmeal and fish oil can be challenging and compromise digestion, absorption, and, consequently, animal growth and health, however, there are some ingredients, that despite being costlier, can be used as alternatives to the normal aquafeed ingredients, because they can help boost the immunity and improve the microbiota dynamics, as seen in **Chapter V**.

The results from the present Thesis combined together contributed to a better understanding of the intestinal inflammatory process on fish and shrimp and its systemic consequences; to a better comprehension on how different ingredients and additives can be used as mechanisms to minimize the development of intestinal inflammation, to help in the recovery of an intestinal inflammatory process; and to improve animal's health status. Nevertheless, further studies are needed to fully understand the intestinal inflammatory process and its consequences and to stablish various means to prevent or treat inflammation.

Keywords: intestinal inflammation, antibiotics, DSS, feed additives, innate immunity, fish, shrimp

Resumo

A aquacultura é uma das atividades agroindustriais com uma maior taxa de crescimento em todo o mundo, no entanto, os surtos de doenças e a escalada dos preços dos ingredientes têm sido um obstáculo ao crescimento contínuo e à sustentabilidade da aquacultura. Por um lado, temos o uso de antibióticos para controlar patógenos e doenças infeciosas que podem desencadear muitas preocupações ambientais e de saúde; por outro lado, o uso de ingredientes não convencionais para substituir a farinha de peixe e o óleo de peixe, podem prejudicar a saúde dos peixes e camarões.

A nutrição desempenha um papel fundamental no crescimento e saúde das espécies aquícolas, sendo o intestino o órgão primordial. O intestino é um órgão muito complexo, não sendo apenas responsável pela digestão e absorção de nutrientes, como também pela modulação do sistema imunológico. Portanto, é de extrema importância entender a sua biologia e fisiologia, uma vez que a saúde intestinal pode influenciar o desempenho geral e a saúde de peixes e camarões. Além disso, como a homeostase intestinal é muito suscetível à manipulação através da dieta, formulações dietéticas alternativas podem ser utilizadas para controlar e melhorar a saúde intestinal e contribuir para peixes mais saudáveis e mais resistentes a patógenos e fatores externos, bem como contribuir para a diminuição de condições inflamatórias originadas pelo uso de ingredientes alternativos.

São escassos os estudos relacionados com os mecanismos associados ao processo inflamatório intestinal em espécies de peixes e camarões, devido à falta de modelos padronizados e robustos que permitam seu estudo. Assim, é de extrema importância estabelecer modelos inflamatórios reversíveis, de baixa ou moderada inflamação, que permitam não só estudar os mecanismos inflamatórios intestinais, como também avaliar a capacidade dos ingredientes da ração em minimizar ou reverter o processo inflamatório e suas consequências sistêmicas.

Desta forma, esta tese de doutoramento pretendeu estudar os efeitos dos antibióticos na saúde intestinal e animal; compreender o impacto de fontes proteicas na recuperação da terapêutica antibiótica; desenvolver um modelo inflamatório intestinal para estudar a inflamação intestinal; e avaliar o efeito de aditivos e ingredientes alimentares promotores da saúde como ferramentas profiláticas.

Nos **Capítulos II**, **III** e **IV** foram avaliados os efeitos nocivos dos produtos químicos na saúde intestinal e dos animais e, embora o antibiótico (oxitetraciclina) usado nos **Capítulos II** e **III** e o produto químico (DSS) usado no **Capítulo IV** produzissem apenas um processo inflamatório intestinal leve, foi possível verificar as modificações produzidas ao nível do perfil de expressão genética, na resposta ao stresse oxidativo e na dinâmica do microbiota

intestinal. Portanto, esses tipos de modelos são de extrema importância para entender o processo inflamatório intestinal e suas consequências.

Também foi possível verificar que as consequências do uso de antibióticos para a saúde (**Capítulos II** e **III**), podem ser revertidas, em certa medida, com diferentes formulações dietéticas, porém, formulações dietéticas extremas podem comprometer o crescimento. Por outro lado, também foi possível utilizar formulações dietéticas alternativas (com aditivos alimentares no caso do **Capítulo IV**) para minimizar os efeitos na saúde dos indutores inflamatórios intestinais (como o químico DSS).

Por outro lado, substituir a farinha de peixe e o óleo de peixe pode ser um desafio e comprometer a digestão, absorção e, consequentemente, o crescimento e a saúde do animal, no entanto, existem alguns ingredientes, que apesar de serem mais caros, podem ser utilizados como alternativas aos ingredientes normais nas rações de aquacultura, uma vez que, podem contribuir para o aumento da imunidade e melhorar a dinâmica do microbiota intestinal, como se verificou no **Capítulo V**.

Deste modo, os resultados obtidos nesta tese, contribuíram para uma melhor compreensão do processo inflamatório intestinal em peixes e camarões e suas consequências sistêmicas e uma melhor compreensão de como diferentes ingredientes e aditivos podem ser utilizados como mecanismos para minimizar o desenvolvimento de inflamação intestinal ou auxiliar na recuperação de um processo inflamatório intestinal e melhorar assim a saúde do animal. No entanto, são necessários mais estudos para entender o processo inflamatório intestinal na sua globalidade e as suas consequências associadas e desenvolver vários meios para prevenir ou tratar a inflamação intestinal.

Palavras-chave: inflamação intestinal, antibióticos, DSS, aditivos alimentares, imunidade inata, peixe, camarão

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List of Abbreviations (Alphabetical order)

ALF	Anti-Lipopolysaccharide Factor
AMPs	Antimicrobial Peptides
AQP10	Aquaporin 10
BACTIN	Beta-Actin
BG	Beta-Glucan
BGBP	Beta Glucan Binding Protein Precursor
BHT	Butylhydroxytoluene
BSA	Bovine Serum Albumin
CASA	Common Agricultural and Wider Bioeconomy Research Agenda
CASP3	Caspase 3
CAT	Catalase
CD8A	Cluster of Differentiation 8 Alpha
cDNA	Complementary DNA
CDNB	Chloro Dinitrobenzene
CFUs	Colony-Forming Units
CSF1R	Colony-Stimulating Factor Receptor
Ct	Cycle Thresholds
CUR	Curcumin
DAMPs	Damage-Associated Molecular Patterns
DMSO	Dimethyl Sulfoxide
DSS	Dextran Sodium Sulphate
ELISA	Enzyme-Linked Immunosorbent Assay
ERDF	European Regional Development Fund
FABP2	Fatty Acid Binding Protein 2
FAO	Food and Agriculture Organization
FM	Fishmeal
FO	Fish Oil
G6PDH	Glucose-6-Phosphate Dehydrogenase
GPX	Glutathione Peroxidase
H-E	Hematoxylin-Eosin
HEP	Hepcidin
HSP70	Heat Shock Protein 70
HT	Haematocrit
IAP	Inhibitor of Apoptosis Protein
IGM	Immunoglobulin M
IL10	Interleukin 10
IL1B	Interleukin 1 Beta
IMD	Immune Deficiency
KO	Krill Oil
KPB	K Phosphate Buffer
L-DOPA	L Dihydroxyphenylalanine
LPS	Lipopolysaccharides

LZC	Lysozyme C
MCH	Mean Corpuscular Haemoglobin
MCHC	Mean Corpuscular Haemoglobin Concentration
MCV	Mean Corpuscular Volume
mRNA	Messenger Ribonucleic Acid
MT1	Metallothionein 1
MTT	Dimethyl Diphenyl Tetrazolium Bromide
MUC13	Mucin 13
MUC2	Mucin 2
NADPH	Nicotanimide Adenine Dinucleotide Phosphate
NGS	Next-Generation Sequencing
NO	Nitric Oxide
NOS	Nitrogen Reactive Species
OCLN	Occludin
OD	Optical Density
OTC	Oxytetracycline
OTU	Operational Taxonomic Units
PAMPs	Pathogen-Associated Molecular Patterns
PCR	Polymerase Chain Reaction
PEN3	Penaeidin 3
PCNA	Proliferating Cell Nuclear Antigen
PO3	Phenoloxidase 3
PPAE	Prophenoloxidase Activating Enzyme
PPM	Parts per Million
PRRs	Pattern Recognition Receptors
QIIME	Quantitative Insights into Microbial Ecology
RBC	Red Blood Cells
ROS	Reactive Oxygen Species
SBM	Soybean Meal
SCAR	Standing Committee on Agricultural Research
SOD	Superoxide Dismutase
TBARS	Thiobarbituric Acid-Reactive Substances
TCBS	Thiosulfate Citrate Bile Salts Sucrose
TETRA	Tetraspanin
TGFB	Transforming Growth Factor Beta
TNFA	Tumour Necrosis Factor Alpha
TRBP1	Tar RNA-Binding Protein 1
TRX2	Thioredoxin 2
TRYP	Trypsin
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
UV	Ultraviolet
WBC	White Blood Cells

Chapter I

General Introduction

General Introduction

1. Aquaculture intensification

Fish are among the healthiest, nutritious, and sustainable protein sources. Fish provide essential nutrients to support human life and good health, such as quality animal proteins, long-chain omega-3 fatty acids, iodine, vitamin D, calcium, zinc, and other micronutrients (Mohanty et al., 2019). According to the Food and Agriculture Organization (FAO, 2020), from 1961 to 2017, the average annual growth rate of fish consumption was 3.1%, with the per capita food fish consumption being 20.5 kg in 2018. In the same year, the global capture fisheries production reached 96.4 million tonnes. However, in 2017, some countries presented overfished stocks, with only 65.8% within the biologically sustainable levels, against the 90% verified in 1990.

As the oceans cannot naturally provide the growing demand of fish, the sustainability of the world´s fish supply has been maintained by the aquaculture sector, reducing the use of wild fish and its environmental impacts. Therefore, the aquaculture sector has been the main available source of fish for human consumption, enhancing fish availability to countries with limited accessibility, providing cheaper prices and more food security. Consequently, the aquaculture sector has significantly expanded in the past decades (Naylor et al., 2021).

According to FAO (2020), the global aquaculture production increased by more than 527 % from 1990 to 2018, consequently to the continuous growth of food fish consumption (more than 122 % in the same period). Records of 82.1 million tonnes of fish production and 114.5 million tonnes in live weight were verified in 2018 (Figure 1). Among the producing countries, China, India, Indonesia, Vietnam, Bangladesh, Egypt, Norway, and Chile were the leaders.

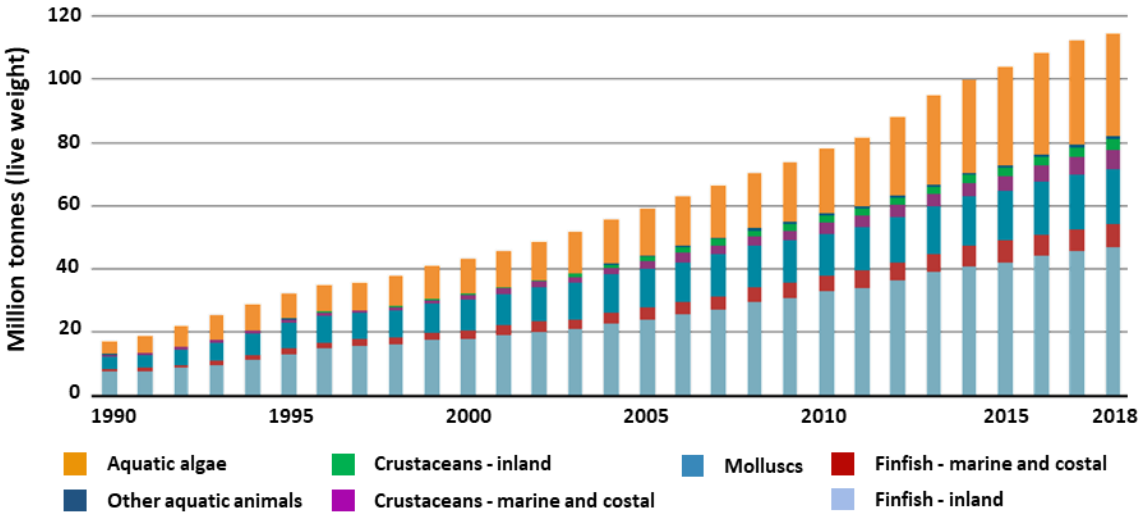

Figure 1. World aquaculture production from 1990 to 2018. A record of 114.5 million tonnes in live weight was achieved in 2018 and the finfish dominated the aquatic animals farming. Adapted from FAO (2020).

Aquaculture industry has grown with new investment, improved technologies, large-scale dedicated engineering developments, automation and mechanisation, selective breeding programmes, improved management practices, and feed technology (FAO, 2017). The world´s fish production is expected to increase from 179 million tonnes in 2018 to 204 million tonnes in 2030. Aquaculture production is likely to reach 109 million tonnes in 2030, with an increase of 32% (26 million tonnes). About 88% of the total fish production is used for human consumption, however, a significant proportion of world fisheries production is processed into fishmeal and fish oil, as fishmeal and fish oil are still considered the most nutritious and digestible ingredients to feed farmed fish (FAO, 2020).

Despite its remarkable development, the aquaculture sector faces serious challenges related to its environmental and social sustainability (Naylor et al., 2021). Social conflicts for land and aquatic resources, habitat destruction, the use of harmful chemicals, the inefficient or unsustainable production of fishmeal and fish oil, and diseases outbreaks are some of the aquaculture vulnerabilities (Carpenter, 2019; FAO, 2020).

2. Aquaculture vulnerability to diseases outbreaks and antibiotic-related risks

The aquaculture industry is extremely vulnerable to environmental stressors (Naylor et al., 2021). Indeed, high stocking densities, poor water conditions and handling practices, distress, stimulate the propagation of pathogenic viruses, bacteria, and parasites (Lulijwa et al., 2019). Hence, as aquaculture has developed, disease outbreaks pose as one of the major threats to its sustainability, as aquatic pathogens spread rapidly, originating huge economic losses (FAO, 2020; Naylor et al., 2021). It is estimated that in some aquaculture industries, the economic impact of infectious diseases is responsible for at least 40% of their global costs (Carpenter, 2019; Shinn et al., 2018).

Shinn et al. (2018) reviewed the cost of diseases in aquaculture, with a focus on the three most valuable aquaculture industries, whiteleg shrimp (*Penaeus vannamei)*, Atlantic salmon (*Salmo trutta)*, and Nile tilapia (*Oreochromis niloticus*). He found record losses of 65% in Atlantic salmon (*Salmo truta*) due to *Aeromonas salmonicida*, mortalities of 90% caused by *Streptococcus agalactiae* in Nile tilapia, and losses of 90% caused by *Hepatobacter penaei* in whiteleg shrimp. In 2020, up to 129 disease outbreaks were reported in Europe, mainly caused by bacterial pathogens, such as those from genus *Aeromonas, Vibrio, Yersinia, Pseudomonas,* and *Photobacterium* (EURL, 2020). Therefore, intensive aquaculture still relies on the use of antibiotics for therapeutic purposes (Lulijwa et al., 2019).

Though antimicrobial agents are essential therapeutic tools for treating and controlling bacterial infections, they have been associated with human, animal, and environmental problems, as they accumulate in the environment, changing the microbial community and

inducing the emergence of antibiotic-resistant species; compromise aquaculture products safety, inducing drug adverse reactions, and chronic toxicity; and induce occupational health hazards in farmers such as allergies or toxicity (da Costa et al., 2013; Watts et al., 2017; Haygood & Jha, 2018; Okocha et al., 2018; Lulijwa et al., 2019; Preena et al., 2020; Silva et al., 2021; Thiang et al., 2021).

According to Lulijwa et al. (2019) and Schar et al. (2020), although there was a drastic reduction in antibiotic use in the last years, due to vaccination and improved husbandry practices, the use of antibiotics is expanding rapidly in the aquaculture industry and is estimated to increase by 33% in 2030 (Figure 2). Catfish, trout, tilapia, shrimp, and salmon are among the species where antibiotics are mostly used, and quinolones, tetracyclines, amphenicols, and sulphonamides are the most used antimicrobial classes worldwide. China, India, Indonesia, and Vietnam are the highest antimicrobial consumer countries in the world.

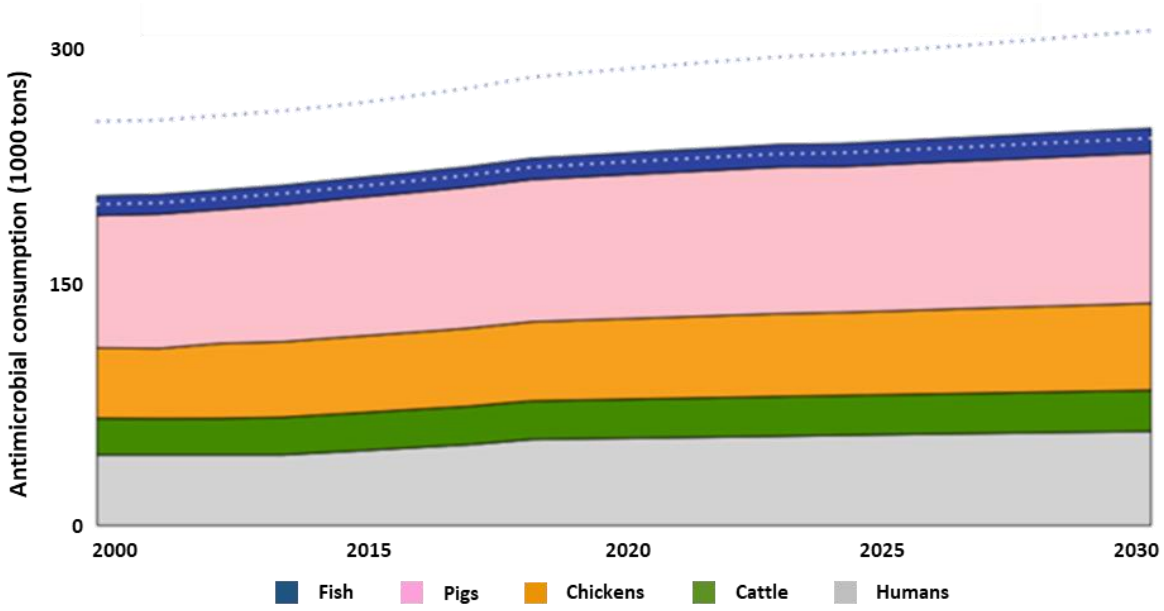

Figure 2. Global antimicrobial consumption from 2013 to 2030. The 95% of uncertainty in the fish interval is represented by the dotted lines. Adapted from Shar et al. (2020).

However, as reviewed by Lulijwa et al. (2019), Schar et al. (2020) and Tiseo et al. (2020), there is an uncertainty about the amounts of antimicrobials used because surveillance networks for monitoring antimicrobial sales have been introduced only recently. So far, only the Japanese aquaculture has successfully banned the use of antibiotics, with no reports of antibiotic use in their aquaculture industry (Lulijwa et al., 2019). In Europe, the aquaculture industry faces strong regulations for the antibiotic use, with a consequent reduction through the years. Antibiotics as growth promoters in animal feed started to be banned in Europe in 2003 (Regulation (EC) No 1831/2003) and are banned since 2006 (European Union, 2005;

Millet & Maertens, 2011). Outside of Europe, notwithstanding genuine efforts for implementing regulation mechanisms of antibiotics use in aquaculture, a significant number of studies carried out in Vietnam, Korea, Brazil, and China have revealed that some classes of antibiotics exceed the country-specific maximum residue limits, and, despite some classes of antibiotics were banned for use in some countries, their illegal use has been reported (Lulijwa et al., 2019, Schar et al., 2020; Tiseo et al., 2020).

Globally, quinolones and tetracyclines accounted for 27% and 20%, respectively, of antimicrobial drugs consumption in animals raised for food. In the aquaculture industry, oxytetracycline is the most used antibiotic (73%) among the major American and European aquaculture-producing countries due to its wide-spectrum, commonly availability and low price (G Rigos & Troisi, 2005; Lulijwa et al., 2019). Oxytetracycline is produced by *Streptomyces* spp. fungi, and limits bacterial growth (Chopra & Roberts, 2001; G Rigos et al., 2003; G Rigos & Troisi, 2005; Yang et al., 2020). It diffuses through the bacterial cell membrane pores, due to its hydrophilic capacity, and interferes with protein synthesis. This antibiotic is commonly administrated in a dose of 75 mg kg^{-1} body weight day^{-1}, however, its administration can vary from 50 - 100 mg kg^{-1} body weight day^{-1}, for 7-10 days, depending on the fish species and route of administration (G Rigos & Troisi, 2005; Leal et al., 2018). In Portugal, the recommended dose is 55 mg Kg^{-1} body weight day^{-1} for 7-10 days in medicated feed (DGAV, 2020). The incorporation into the feed is the most common route of administration and is safer for the environment (Sekkin & Kum, 2011). This is a broad-spectrum bacteriostatic antibiotic, designated to treat fish bacterial diseases caused by *Aeromonas salmonicida* (furunculosis), *Aeromonas hydrophila,* and *Aeromonas sobia* (aeromonosis), *Pseudomonas* spp. (pseudomonosis), *Lactococcus garvieae* (lactococcosis) and *Vibrio anguillarum* (vibriosis) (Leal et al., 2018; DGAV, 2020). The main target species are salmonids (e.g. Atlantic salmon and trout), carp (*Cyprinus carpio*), catfish (*Ictalurus punctatus*), tilapia, ayu (*Plecoglossus altivelis*), olive flounder (*Paralichthys olivaceus*), turbot (*Scophthalmus maximus*), gilthead seabream (*Sparus aurata*), European seabass (*Dicentrarchus labrax*), and European eel (*Anguilla anguilla*) (Sekkin & Kum, 2011; Leal et al., 2018; DGAV, 2020). The absorption of oxytetracycline is typically rapid, despite being temperature-related and is poorly metabolized in the fish body; its bioavailability is affected by complex formation with ions (mainly calcium-Ca^{2+} and manganese-Mg^{2+}). Oxytetracycline is released in the environment freely dissolved or associated with organic and inorganic compounds and its by-products seem not to have biological activity (Leal et al., 2018).

Though diet supplementation with nutraceuticals can be used to influence fish disease resistance, optimal management practices are crucial to minimize pathogen occurrence in the production systems. Selective breeding and vaccines can be also used as an alternative to antibiotics, but they are costly and sometimes inefficient in some species. Therefore, the

management of pathogens remains a challenge for the viability and sustainability of the aquaculture industry (Naylor et al., 2021; Schar et al., 2020).

The Common Agricultural and wider bioeconomy reSearch Agenda (CASA) and the Standing Committee on Agricultural Research (SCAR) reviewed the major challenges within the aquaculture sector in Europe and found that disease control and vaccines improvement were selected as extremely important factors. The environmental impact of treatments and antimicrobial resistance were also highlighted as disturbing topics. Microbial control is essential for the aquaculture sector, as many opportunistic pathogens are responsible for mass mortalities, vertical transmission of disease needs to be avoided, and the presence of intracellular bacteria must be regarded as an emerging problem (Katharios, 2019).

3. Aquaculture sustainability with fishmeal and fish oil replacers

Aquatic animals have specific nutritional requirements for health, survival, growth, development, and reproduction. Currently, aquafeeds still rely on fishmeal and fish oil sourced from wild-captured fish, especially for feeding marine shrimps and carnivorous fish (Tables 1 and 2) and the prices of these wild fish-based ingredients have doubled in the last years. Thus, continued reliance on wild fish to produce fishmeal and fish oil restricts the growth of aquaculture and creates a sustainability problem from farmed products (Tacon & Metian, 2008; FAO, 2011; EUFOMA, 2021; Hua et al., 2019; FAO, 2021; Naylor et al., 2021).

Fishmeal	2016	2017	2018	2019
Peru	633	735	1406	796
China	460	400	570	477
Thailand	234	331	377	350
Chile	300	305	308	310
Vietnam	252	243	275	256
Others	2664	2865	2829	2699
Total	4543	4878	5763	4887

Fish oil	2016	2017	2018	2019
Vietnam	155	160	174	189
Chile	81	120	151	133
Peru	114	99	227	126
USA	114	99	227	126
Japan	64	81	75	74
Others	527	564	449	525
Total	1055	1122	1303	1172

Tables 1 and 2. Global production (1000 tonnes) of fishmeal (Table 1) and fish oil (Table 2) from 2016 to 2019. Adapted from FAO, 2021.

Consequently, world aquaculture fish production claims for development and optimization of alternative protein sources for aquafeeds, such as fisheries and aquaculture by-products, processed food waste, insect meals, microbial and macroalgal biomass, among others (FAO, 2011; Naylor et al., 2021; Jia et al., 2022). However, despite the efforts to innovate in alternative protein-sourced feedstuffs, the use of alternative energy and protein sources can affect fish health by altering the intestinal morphology and microbiota, modifying the immune system, interfering with the normal function of the endocrine system, and potentiating disease risk (EUFOMA, 2021; FAO, 2021; Naylor et al., 2021; Jia et al., 2022). Therefore, it is crucial

to understand the effects of fishmeal and fish oil replacement by other protein and lipid sources, as well as to understand the consequent inflammatory conditions associated with the use of these ingredients in intestinal and fish health, especially in carnivorous fish that have difficulty digesting starch, non-soluble carbohydrates, and fiber, and are more sensitive to the antinutrients and toxins present in some alternative ingredients, such as plant protein ingredients. (FAO, 2011; Naylor et al., 2021).

4. Fish and shrimp species in this Thesis

4.1. Nile tilapia (*Oreochromis niloticus*)

Freshwater fish account for 75% of global edible aquaculture volume. The role of freshwater systems has gained attention due to the advances in feed technology and breeding, that are addressing the concerns regarding the effects of aquaculture on wild-capture fisheries. Freshwater aquaculture is widely recognized to produce carp, tilapia, and catfish (Naylor et al., 2021).

Nile tilapia (*Oreochromis niloticus,* Linnaeus 1758) (Figure 3) is the second (8.3%) most produced finfish worldwide, after carps (FAO, 2020). China is the largest producer, followed by the Philippines, Thailand, and Indonesia. Tilapia is an omnivorous species that feeds on phytoplankton, periphyton, aquatic plants, small invertebrates, benthic fauna, detritus, and bacterial biofilms associated with detritus, with a range of temperature of 31 - 36 ºC, and lethal temperatures below 12 ºC or above 42 ºC. It is easily breed and cultured intensively and economically, grows rapidly on formulated feeds with lower protein levels, tolerates higher carbohydrate levels than many carnivorous farmed species, and accepts feeds with a higher percentage of plant proteins (FAO, 2022a).

Figure 3. Nile tilapia (*Oreochromis niloticus*), the dominant tilapia's species cultured worldwide. Photo by Carla Teixeira.

Tilapia culture has benefitted from genetic and nutritional advances; however, remained vulnerable to stock densities, management practices, and climate events. Resources depletion, nutrient pollution, disease problems, and fish mortality have been associated with its culture intensification. Also, tilapia relies on the combination of commercially formulated pelleted feed and naturally occurring nutrients to produce low-cost tilapia in semi-intensive systems (Naylor et al., 2021). A high-quality environment and reduced handling stress are crucial to prevent diseases; however, because *Aeromonas hydrophila*, *Vibrio anguillarum*, *Flavobacterium columnare*, *Edwardsiella tarda*, *Streptococcus iniae,* and *Enterococcus* sp. are commonly implicated in tilapia's infections, antibiotic treatments are sometimes necessary. The antibiotics are mainly applied in medicated feed treatments (FAO, 2022a).

4.2. Whiteleg shrimp (*Penaeus vannmaei*)

The whiteleg shrimp (*Penaeus vannamei*, Boone 1931) (Figure 4) is the most produced crustacean worldwide (FAO, 2020; FAO, 2022b; WoRMS, 2022).

Figure 4. Whiteleg shrimp (*Penaeus vannamei*), the leading crustacean species cultured worldwide. Photo by Carla Teixeira.

Shrimps can be cultured in extensive, semi-intensive, intensive, and super-intensive, with low, medium, high, and extremely high stocking densities, respectively. China, Thailand, Indonesia, and Vietnam are the main producing countries. Whiteleg shrimps are very efficient in utilizing feed and present lower protein requirements than more carnivorous shrimp. Most shrimp diseases are caused by viruses (white spot syndrome virus, taura syndrome virus), and no chemicals and drugs are available to treat these infections; however, good management practices, adequate feed, and a good health status of the stocks can help to reduce the occurrence of diseases outbreaks. Genetic selection programs can also be used to provide more resistant shrimps. *Vibrio harveyi* and *V. parahaemolyticus*, are the two main bacteria that affect the whiteleg shrimp aquaculture. Due to the quick expansion of shrimp aquaculture, and its negative effects on the environment, many organizations are joining efforts to adapt more responsible and sustainable guidelines, and stricter measures are being applied to ban the use of antibiotics in this sector (FAO, 2022b).

4.3. Gilthead seabream (*Sparus aurata*)

Gilthead seabream (*Sparus aurata*, Linnaeus 1758) is mainly produced in the Mediterranean Sea (Greece, Turkey, Spain, and Italy), in extensive, semi-intensive, and intensive farming systems (Figure 5) (Basurco et al., 2011; FAO, 2022c). It is a benthopelagic and a euryhaline fish, with a carnivorous behaviour; however, it can be accessorily herbivorous (Basurco et al., 2011).

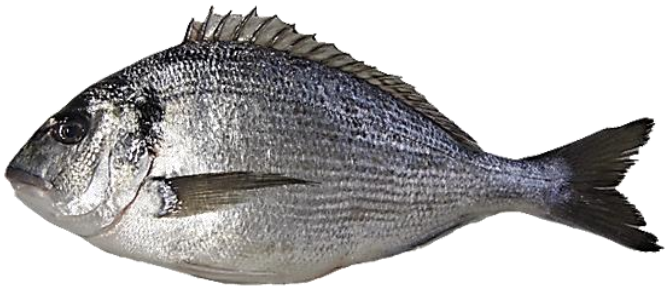

Figure 5. Gilthead seabream (*Sparus aurata*), a common species produced in the Mediterranean Sea. Photo by Lourenço Ramos-Pinto.

Their good market price, high survival rate, and feeding habits make this species very suitable for aquaculture. Breeding programs helped to improve its adaptability to intensive rearing conditions; however, they are very sensitive to low temperatures, with a lethal limit of 4ºC. Many viruses and bacteria (e.g., *Photobacterium damselae*, *Vibrio alginolyticus*, *Vibrio anguillarum,* and *Pseudomonas anguilliseptica*) are the main causative agents of infectious diseases (FAO, 2022c).

5. The intestinal health: structure, immunity, and microbiota

5.1. Teleost fish

The intestine of fish is a multifunctional organ and one of the main physiological barriers against pathogens (Ray & Ringo, 2014). Comprised of an epithelial layer (digestion and nutrient absorption), non-organized lymphoid tissues (immune response) and resident bacteria (microbiota) (Figure 6) (Ray & Ringo, 2014; Merrifield & Rodiles, 2015; Vallejos-Vidal, et al., 2016). The intestinal epithelium is composed of absorptive columnar enterocytes and goblet cells, which secrete a mucous layer responsible for physical and chemical barriers, osmoregulation, and lubrication (Birkbeck & Ringo, 2005; Ray & Ringo, 2014; Merrifield & Rodiles, 2015; Salinas & Parra, 2015; Vallejos-Vidal et al., 2016). Mucins, the proteins secreted by the goblet cells, are released in the intestinal lumen, and maintain the mucosal layer health (Salinas & Parra, 2015). The mucosal layer is more pronounced in the posterior

part of the intestine, as the number of goblet cells increases in this segment, sequentially diminishing its digestive and absorptive functions (Borlongan et al., 2002; Ray & Ringo, 2014). Different enzymes and bile acids are produced by the liver and pancreas and are continuously released in the intestine, to facilitate the digestive process. Their production, which is modulated by diet composition, especially dietary fiber, may affect the growth and dynamics of the intestinal microbiota (Ray & Ringo, 2014).

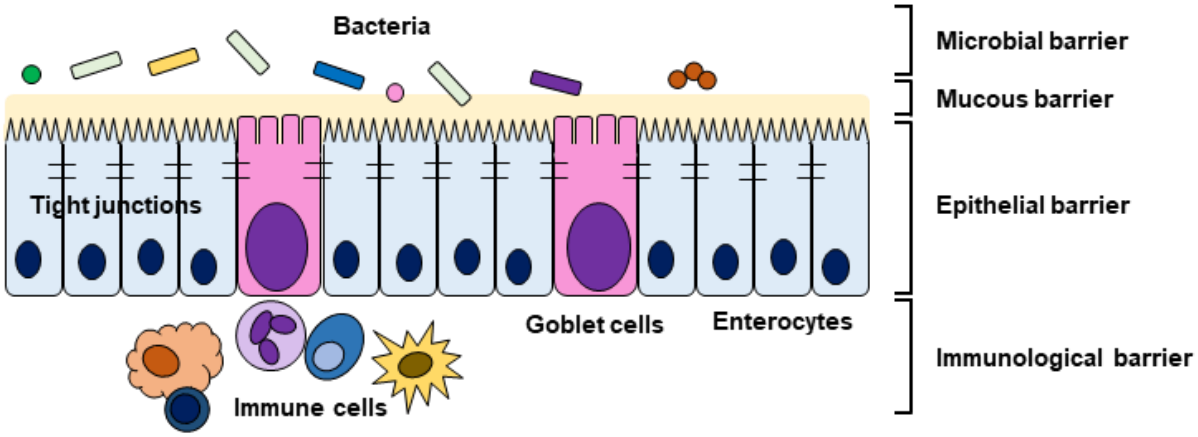

Figure 6. The intestinal barrier. Intestine epithelium structure represented by the main resident cells (enterocytes, goblet cells, macrophages, B cells, T cells and plasma cells), mucus layer and associated microbiota. Original scheme.

The intestinal immune response is regulated by immunological cells (granulocytes, macrophages, lymphocytes, plasma cells, and T and B cells), epithelial cells, mucous-producing cells, and neuroendocrine cells (Vallejos-Vidal et al., 2016). Also, innate and adaptive immune factors (humoral immunity), such as antimicrobial molecules, immunoglobulins, C-reactive protein and lectins, are present in the intestinal mucous to protect the organism against pathogens. Immunoglobulin T is the main immunoglobulin found in the intestine and is responsible for the interaction of the intestinal mucosal barrier with the microbiota, together with IgM which has been shown to coat a significant portion of the microbiota (~12-50%) on different mucosal surfaces (Smith & Fernandes, 2009; Foey & Picchietti, 2014; Salinas & Parra, 2015; Salinas et al., 2021). β-defensins are some of the antimicrobial peptides (AMPs) present in the intestinal barrier. Some AMPs break down the bacterial cell-wall, destroying them or preventing their multiplication (Smith & Fernandes, 2009; Foey & Picchietti, 2014; Salinas & Parra, 2015).

If this barrier is compromised, the innate immune cells more involved in phagocytosis (macrophages and neutrophils) are activated, beginning the pathogen opsonization, activating the respiratory burst, and ultimately initiating the inflammatory process through the expression of cytokines (Figure 7). The activation of these cells begins with the detection of pathogens

through pattern recognition receptors (PRRs) that bind to the bacteria-associated molecular patterns (PAMPs) expressed by the pathogen. The phagocytosis process results in the release of reactive oxygen species as part of the respiratory burst that destroys the pathogenic bacteria. Lectins and complement components are also involved in opsonization, phagocytosis, chemotaxis, inflammation and pathogen destruction. The cytokine signalling amplifies the innate immune response, inducing the chemotaxis of immune cells, regulating inflammation, stimulating the respiratory burst, and helping in the destruction of pathogens (Belosevic et al., 2009; Foey & Picchietti, 2014).

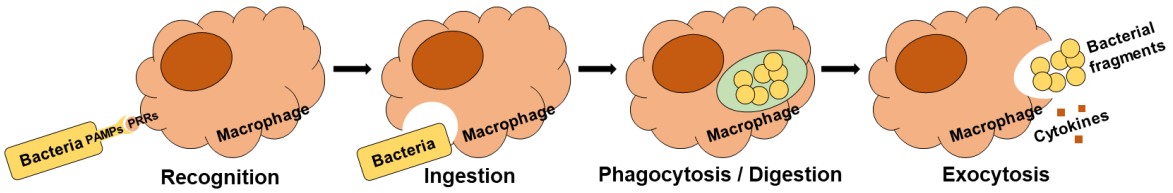

Figure 7. Pathogen recognition, phagocytosis and destruction. The phagocyte recognizes pathogens by specialized receptors on its cell surface (PRRs) that binds to specific molecules on the cell surface of the pathogen (PAMPs) to initiate the innate immune response. Original scheme.

When present, the adaptative immunity helps to improve the immune response towards pathogens. It is a specific antigen response, with slower development and memory-adaptation in re-exposure cases. The expression of the major histocompatibility complex and immunoglobulins, as well as the presence of T and B lymphocyte cells, are associated with this adaptative immune response. They produce a well-adapted and antigen-specific response towards a type of pathogen, helping the innate immune effectors in its destruction. Several T-cell lymphocyte populations (such as helper and cytotoxic) are present in the intestine, integrated with the cell-mediated cytotoxic immunity, humoral immunity, homeostasis, and regulatory responses. B-cell lymphocytes produce antibodies against specific pathogens and are activated by T-cells, cytokines, and PAMPs. These antibodies or immunoglobulins are involved in precipitation, agglutination, neutralization, opsonization, and complement activation, conferring humoral immune protection. The cytokine signalling molecules (such as interleukins, tumour necrosis factors, transforming growth factors, and interferons) and their profile are responsible for the development, activation, and functionality of T and B lymphocytes (Buonocore & Scapigliati, 2009; Foey & Picchietti, 2014).

The resident intestinal microbiota (autochthonous) is a complex community that establishes a balanced homeostasis with external microorganisms (allochthonous), the internal structure, and the immune system, providing nutritional and metabolic support (Salinas & Parra, 2015; Egerton et al., 2018). This balance and stability are related to microbiota complexity and richness, as higher diversity provides a better opportunity to control pathogens and potentiate

symbiotic functions (Vargas-Albores et al., 2021). Consequently, a diverse microbiota is usually found in healthy fish, and a low diversity microbiota is associated with diseased fish (T Li et al., 2016; Nie et al., 2017; T Li et al., 2017; Xiong et al., 2019; Bozzi et al., 2021; Vargas-Albores et al., 2021).

Commensal bacteria compete with gut pathogens (bacteria-bacteria interactions) by efficient utilization of available nutrients, production of metabolites and antimicrobial compounds, creation of a physicochemical microenvironment unsuitable for pathogen colonization, competition for epithelial receptors, and occupying the ecological sites within the intestine to competitively exclude colonization of pathogenic bacteria (Bauer et al., 2018). Additionally, intestinal microbiota also influences the host (host-bacteria interactions), playing an important role in the modulation of the innate immune response, nutrient digestion, and regulation of intestinal function, by affecting mucin synthesis and mucosal cell proliferation and maturation (Rawls et al., 2004; Nayak, 2010; Foey & Picchietti, 2014; Wang et al., 2018; Butt & Volkoff, 2019; Xiong et al., 2019; Fuess et al., 2021; Naya-Catala et al., 2021). Also, the normal gut microbiota influences the overall performance of fish, since these microorganisms provide complementary enzymatic activities and nutritional products (Sugita et al., 1990; Ramirez & Dixon, 2003; Banerjee et al., 2013; H Liu et al., 2016; Wang et al., 2018; Xiong et al., 2019; Dhayalan et al., 2022).

In mammals, Bacteroidetes and Firmicutes are the dominant phyla, whereas Proteobacteria and Firmicutes seem to be the prominent phyla in fish, although numerous factors, such as fish species and age, culture system, intestinal portion and diet, influence the microbiota composition (Romero et al., 2014; Merrifield & Rodiles, 2015; Egerton et al., 2018; Wang et al., 2018). *Vibrio*, *Photobacterium*, *Clostridium*, *Pseudomonas*, *Bacillus*, and *Micrococcus* are the most reported genera found in fish microbiota and are associated with pathogenic (disease) and beneficial (e.g., probiotic, production of hydrolytic enzymes, symbiotic) species (Egerton et al., 2018; Wang et al., 2018). The anaerobic bacteria most described in the fish intestine belong to the *Clostridium* and *Bacteroides* genera (Romero et al., 2014). *Clostridium* seems to be linked to herbivorous, while *Vibrio* and *Photobacterium* are more commonly found in carnivores animals (Egerton et al., 2018). On the other hand, bacterial diversity seems to be more prominent in herbivorous and omnivorous animals than in carnivorous ones (He et al., 2013; Romero et al., 2014; H Liu et al., 2016). Also, the microbiota of herbivorous fish can break undigestible fibers, ferment carbohydrates into short-chain fatty acids and produce microbial by-products, providing numerous nutrients for the development and growth of fish (Romero et al., 2014; Haygood & Jha, 2018). A preference for a type of environment (fresh or marine water) was also described, with *Vibrio*, *Pseudomonas*, *Acinetobacter*, *Corynebacterium*, *Alteromonas*, *Flavobacterium*, and *Micrococcus* being the dominant genera in marine fish microbiota, whereas *Aeromonas*, *Pseudomonas*, and

Bacteroides are the main genera found in freshwater fish (Romero et al., 2014; Wang et al., 2018).

5.2. Shrimp

The gastrointestinal tract of penaeid shrimps is formed by the foregut (oesophagus and stomach), the midgut (hepatopancreas and intestine), and the hindgut (rectum and anus) (Borlongan et al., 2002; Holt et al., 2021). The intestine has a chitinous lining, the peritrophic membrane, that protects the intestinal epithelium from pathogens; however, when breached, pathogens can reach the epithelium and initiate a humoral and cellular innate immune response (Borlongan et al., 2002; Kulkarni et al., 2020). The natural immunity of shrimp is very effective against pathogens, and came through haemocytes (hyalinocytes, semi-granulocytes and granulocytes), plasma components (antimicrobial peptides, lysosomal enzymes, recognition molecules), and multimeric systems (clotting protein cascade, prophenoloxidase system) (Figure 8) (Aguirre-Guzman et al., 2009; Kulkarni et al., 2020).

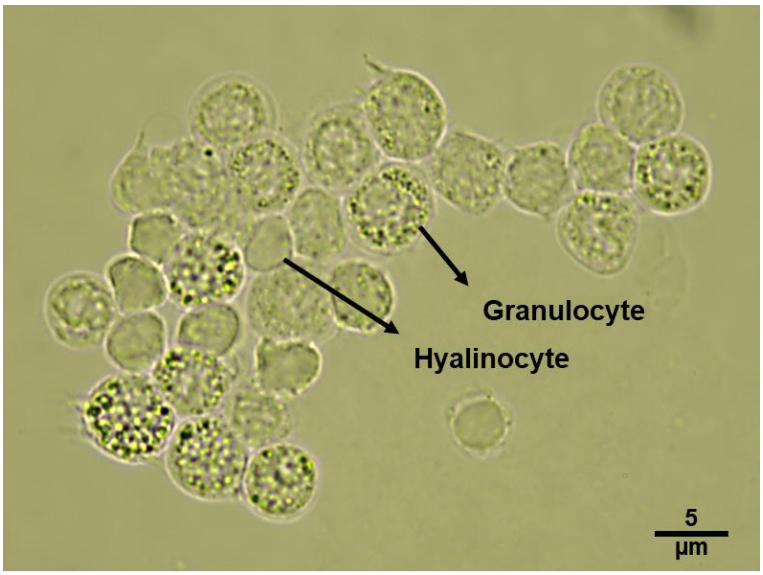

Figure 8: Types of haemocytes found in the haemolymph of *Penaeus vannamei* in light microscopy. Hyalinocytes (associated with clotting) and granulocytes (associated with phagocytosis and the prophenoloxidase system). Photo by Carla Teixeira.

The open circulatory system of crustaceans allows haemolymph circulation through the tissues, transporting haemocytes and humoral components. Haemocytes present the same biological functions that fish macrophages, granulocytes, and natural killer cells, participating in phagocytosis, encapsulation, nodule formation, wound repair, clotting and prophenoloxidase activation. Haemocytes also produce adhesion molecules, agglutinins, and antimicrobial peptides (Aguirre-Guzman et al., 2009).

Though shrimps do not possess a true adaptive immune system, the highly effective innate immune response is based on non-self-recognition mechanisms for microbial detection. This pathogen recognition in based in PRRs that recognizes PAMPs, but also through the detection of certain danger signals associated with cellular stress, known as damage-associated molecular patterns (DAMPs). The activated PRRs cells originates the expression of pro-inflammatory cytokines, the production of antimicrobial molecules, triggering a series of cellular or humoral responses such as the prophenoloxidase-activating system (proPO), clotting mechanism, phagocytosis, and the release of NF-kB dependent AMPs (Figure 9) (Kulkarni et al., 2020).

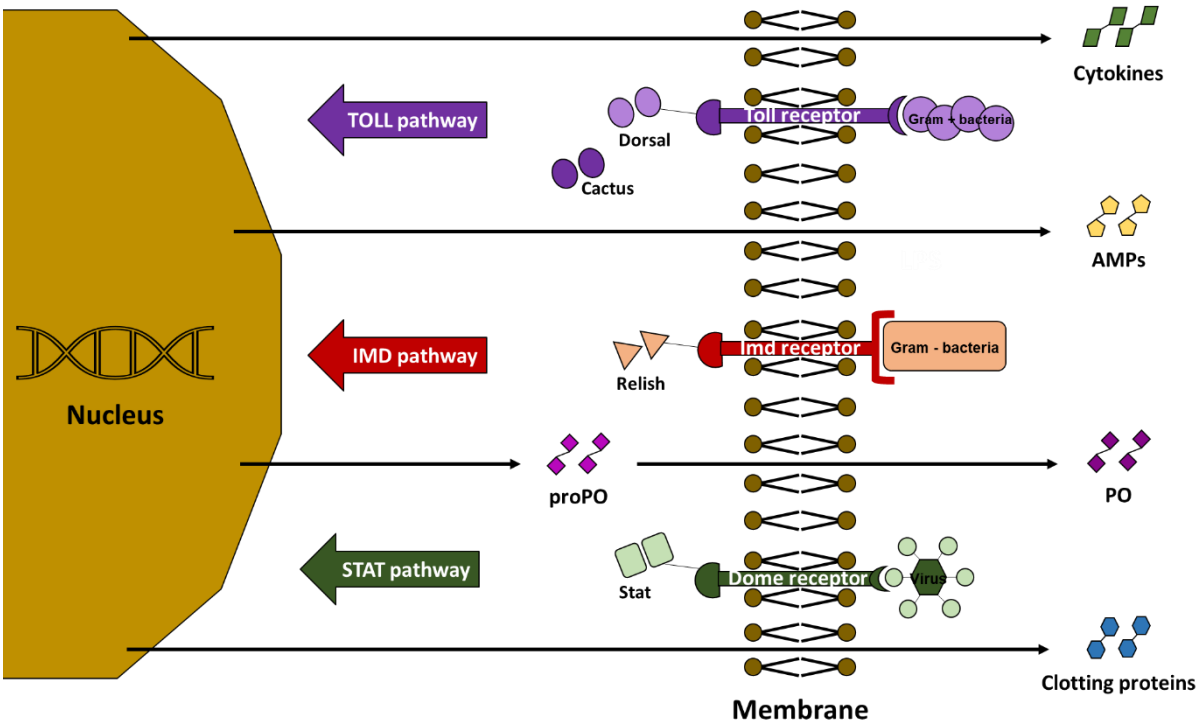

Figure 9. Pathogen recognition and regulatory signalling pathways in shrimp. In response to microbial recognition, different signalling cascades are activated, triggering a series of cellular or humoral responses. Original scheme.

The phagocytosis process in shrimps also involves chemotaxis, adherence, ingestion, pathogen destruction and exocytosis (Aguirre-Guzman et al., 2009). When the microorganism or antigen is bigger and cannot be remove by phagocytosis, encapsulation and nodulation are initiated, with numerous haemocytes appearing around the foreign agent (Aguirre-Guzman et al., 2009; Kulkarni et al., 2020). The prophenoloxidase system is triggered by the presence of β-glucans, peptidoglycans, and lipopolysaccharides, with granulocytes activating the prophenoloxidase enzymes to oxidize phenols into quinones to destroy the invading organism or produce melanin. Melanin is organized in acellular capsules, that involves and inhibits the foreign agent due to its microbicidal properties. The clotting system is activated when the

shrimp´s exoskeleton is damage to prevent haemolymph loss or to immobilize pathogens (Aguirre-Guzman et al., 2009; F Li & Xiang, 2013). The hemocyanin is a protein complex, and the major component of the haemolymph, being responsible not only for the transport of oxygen but also for contributing to the immune system (F Li & Xiang, 2013).

The innate immune responses of shrimp are mediated by cytokines, pattern recognition proteins and antimicrobial peptides (Aguirre-Guzman et al., 2009). Pattern recognition proteins, such as lectins, detect the invading organism and activates the defence mechanisms, generating an inflammatory response (Aguirre-Guzman et al., 2009; Kulkarni et al., 2020; F Li & Xiang, 2013). Because shrimp and other invertebrates lack an adaptative immunity, the antimicrobial peptides, such as penaeidins, crustins and antilipopolysaccharide factor, are crucial against pathogens (Aguirre-Guzman et al., 2009). They are synthetized and stored in granulocytes, having the ability to kill or slowdown the growth of microorganisms, with a wide spectrum activity, low specificity and weak cytotoxic capacity against the host cells (Aguirre-Guzman et al., 2009; Bilej, 2015; F Li & Xiang, 2013). Lysozyme, an antibacterial enzyme, with hydrolytic activity towards bacterial peptidoglycan, helps in the recognition and degradation of microorganisms (Aguirre-Guzman et al., 2009; Bilej, 2015). Despite the absence of an acquired immune response, a form of immune memory, also known by innate immunity with specificity, has been described in shrimp (Kulkarni et al., 2020).

The intestinal microbiota of shrimps plays a huge role on shrimp's health and performance, helping the host to improve the immune response, to increase the nutrient absorption, and to maintain the homeostasis (E Li et al., 2018). This microbiota seems to be characterized by the presence of *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* phyla, with *Photobacterium*, *Vibrio*, and *Aeromonas* being the dominant genera (Holt et al., 2021; E Li et al., 2018). In healthy shrimp intestines, both beneficial bacteria and opportunistic pathogenic bacteria co-exist. *Pseudomonas*, *Flavobacterium*, *Escherichia*, *Aeromonas*, *Vibrio*, *Rickettsia*, *Shewanella*, and *Desulfovibrio*, are some of the opportunistic pathogens that can be found in shrimp's intestines. Normally these bacteria are present with low abundance, however, in stressful conditions, they can multiply and induce disease outbreaks (E Li et al., 2018).

6. The intestinal disease: dysbiosis and inflammation

The gut microbiota establishes an equilibrium with the host and the external environment; however, fluctuations are frequently observed (Vargas-Albores et al., 2021). Naturally, oscillations in the intestinal microbiota are related to the species, sex, developmental stage, diet, water parameters, and season, and they are not usually harmful (W Li et al., 2018; Piazzon et al., 2019; Xiong et al., 2019; Vargas-Albores et al., 2021). They contribute to the development of the host, as the intestinal microbiota is selected and reinforced with time

(Xiong et al., 2019). On the other hand, microbiota pathological variations, due to extreme diets, antibiotics and other chemicals, and genetic or acquired diseases can generate an imbalance in the dynamic of the intestinal community. This can lead to a decrease in bacterial diversity and overgrowth of pathogenic bacteria, which induces dysbiosis and compromises the intestinal health (Hawrelak & Myers, 2004; Pindling et al., 2018; Qiao et al., 2019; Xiong et al., 2019; Bao et al., 2020; Kakade et al., 2020; Roy Choudhury et al., 2021; Vargas-Albores et al., 2021). Many antibiotics used in aquaculture are of broad-spectrum activity, affecting the viable numbers and diversity of the intestinal microbiota (Romero et al., 2014; Pindling et al., 2018; Kim et al., 2019; Legrand et al., 2020). They eradicate the susceptible microorganisms and potentiate the proliferation of resistant opportunistic pathogens by reducing bacterial competition and enabling the colonization of the newly available biding sites (Romero et al., 2014; He et al., 2017b; Legrand et al., 2020). Moreover, dietary antigens, anti-nutritional factors, and dietary bacteria can potentiate inflammatory processes and release toxins that, subsequently, contribute to dysbiosis and intestinal inflammation (Hawrelak & Myers, 2004; Ingerslev et al., 2014; Batista et al., 2016; Lopez Nadal et al., 2018; Tran et al., 2018; Perry et al., 2020). These are closely related, as dysbiosis may contribute to or result from intestinal inflammation (Belizario et al., 2018; Amoroso et al., 2020; S Xie et al., 2020; Orso et al., 2021). There is also a close relationship between nutritional status, immune function, and disease resistance as dietary factors are related to the maintenance of epithelial barriers (Birkbeck & Ringø, 2005). Disease potentiates the susceptibility to dysbiosis, due to a depressed immunity and a decrease in beneficial bacteria (Tran et al., 2018; Bozzi et al., 2021; Vargas-Albores et al., 2021).

To break the intestinal barrier, pathogens need to survive the gastric transit, compete and survive the intestinal commensal microbiota, colonize the intestinal mucous and break the intestinal structure. They express adhesins that allow the anchorage to the intestinal cells (Birkbeck & Ringø, 2005; Ringø et al., 2007; Patel et al., 2017). When attached, pathogens produce toxins that aggressively damage the intestinal wall (Ringø et al., 2007). Ultimately, these pathogens migrate from the intestine to other organs and establish a systemic infection (Figure 10) (Ringø et al., 2007; Bøgwald & Dalmo, 2014; Mosberian-Tanha et al., 2016; Xiong et al., 2019).

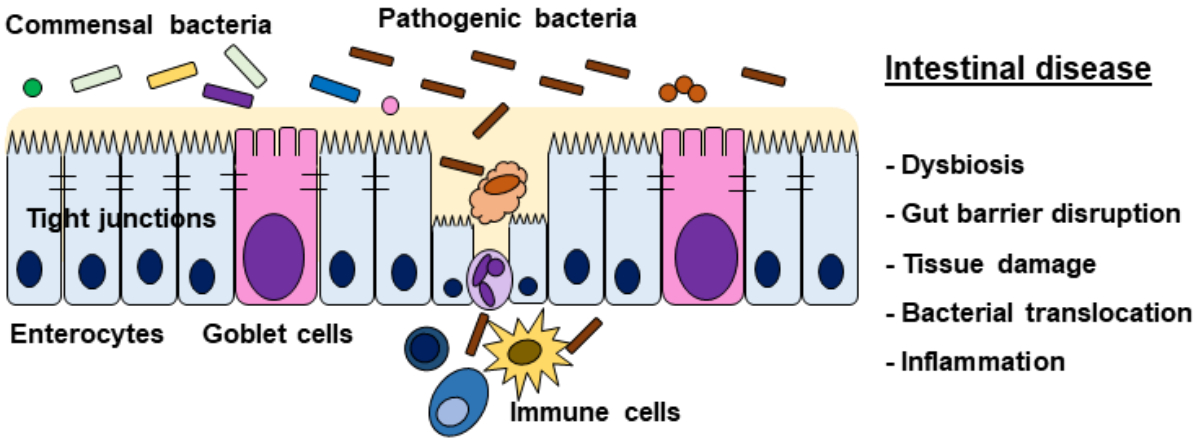

Figure 10. Dysbiosis, inflammation, and intestinal disease. The intestinal dysbiosis promoted by high numbers of pathogenic bacteria, disrupts the tight junction integrity. This loss of integrity makes the epithelium more permissive to microbes and other metabolites that enter the circulation and contribute to the intestinal inflammation. Original scheme.

Vibrio (*V*. *anguillarum*, *V*. *salmonicida*, *V*. *vulnificus*, *V*. *ichthyoenteri*, V. *harveyi*), *Aeromonas* (*A*. *salmonicida*, *A*. *hydrophila*), *Yersinia* (*Y*. *ruckeri*), *Edwardsiella* (*E*. *ctalurid*, *E*. *tarda*), *Piscirickettsia* (*P*. *salmonis*), *Pseudomonas* (*P*. *anguilliseptica*), *Photobacterium* (*P*. *damsela* subsp. *Piscicida*), *Lactococcus* (*L*. *garvieae*, *L*. *piscium*), *Streptococcus* (*S*. *iniae*, *S*. *agalactiae*, *S*. *parauberis*), *Enterococcus* spp., *Vagococcus* (*V*. *salmoninarum*), *Candidatus* (*C*. *arthromitus*), *Mycobacterium* (*M*. *marinum*, *M*. *peregrinum*, *M*. *chelonae*), are some of the pathogens responsible for causing intestinal bacterial diseases in fish and shrimp (Birkbeck & Ringø, 2005; Bøgwald & Dalmo, 2014) (Birkbeck & Ringø, 2005; Bøgwald & Dalmo, 2014).

7. Enhancing fish and shrimp health through fish nutrition: immune boosting, intestinal strengthening, microbiota improvement, and inflammation management

The increase of drug-resistant pathogens and the environmental and food safety concerns related to the use of antibiotics and other chemicals in aquaculture imposes the urgent need to find more natural and eco-friendly therapeutic approaches to disease outbreaks (Vallejos-Vidal et al., 2016). Nutrition plays an important role in fish growth and health, as amino acids, fatty acids, vitamins, and minerals are essential nutrients for fish to obtain energy and produce structural components (M Hixson, 2014). As a multifunctional organ involved in fish nutrition and immunity, the intestine can be manipulated through diet, using functional feeds, to alleviate stress effects and boost the immune system, decrease susceptibility to pathogens and increase disease resistance, strengthen the intestinal functions and health, improve the microbiota community and manage the inflammatory processes (Figure 11) (Egerton et al.,

2018; Haygood & Jha, 2018; M Hixson, 2014; Perry et al., 2020; Salinas & Parra, 2015; Vallejos-Vidal et al., 2016).

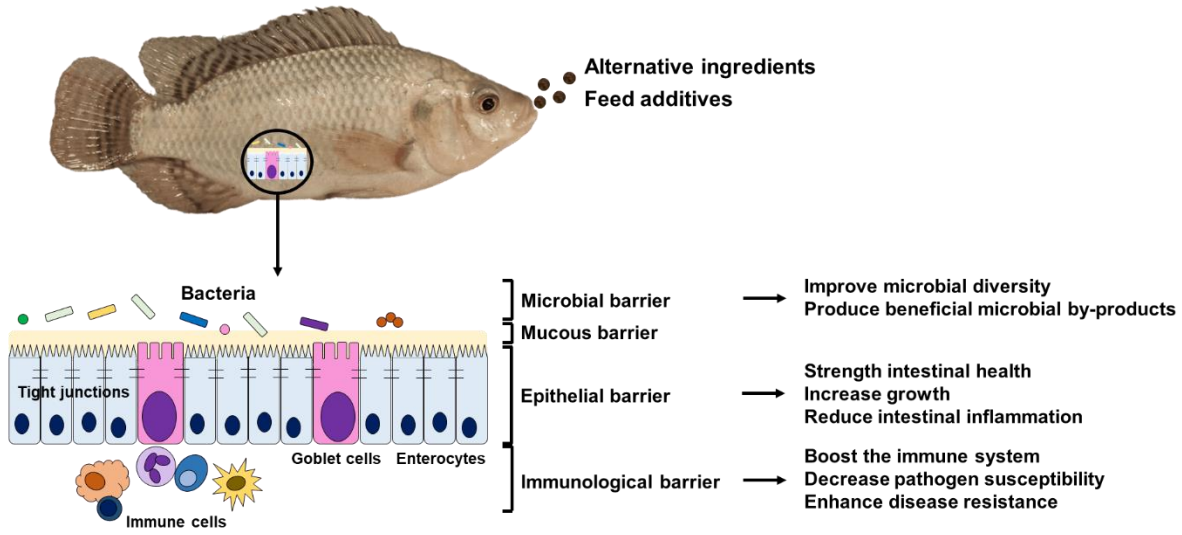

Figure 11. Improving intestinal and fish health with functional diets. Original scheme.

The dietary inclusion of drugs or vaccines, the integration of beneficial microbes (probiotics), and the incorporation of specific nutrients (alternative feed ingredients, immunostimulants, and prebiotics) can help to improve the fish health condition, feed efficiency, growth performance, immunity, stress tolerance and disease resistance (Salinas & Parra, 2015; Vallejos-Vidal et al., 2016; Haygood & Jha, 2018). Vaccination is a prophylactic measure to improve immune functioning and disease resilience in farmed fish (L Liu et al., 2015; Sudheesh & Cain, 2017; Ma et al., 2019). Probiotics are live microbial supplements that beneficially affect the host-microbial balance, and prebiotics are nondigestible ingredients that stimulate the growth of beneficial bacteria (Akhter et al., 2015; Haygood & Jha, 2018; Vargas-Albores et al., 2021). *Bacillus* and *Lactobacillus* are two common genera of probiotics tested in aquaculture (Topic Popovic et al., 2016; He et al., 2017a; Soltani et al., 2019; Tarnecki, et al., 2019; Olmos et al., 2020; Perry et al., 2020). Alternative feed ingredients are unconventional ingredients usually derived from plant, animal, or microbial sources, that are rich in fiber content and act similarly to prebiotics (Haygood & Jha, 2018; Vargas-Albores et al., 2021). Immunostimulants are substances that stimulate the fish immune system by interacting with the mucosal tissues, activating local physiological responses that induce effects at a systemic level, with a minimal impact on the environment and the consumer (Ringø et al., 2011; Vallejos-Vidal et al., 2016). Some examples are β-glucans and curcumin that have antioxidant, anti-inflammatory, antimicrobial, and immunomodulatory properties (Ali et al., 2006; Meena et al., 2013; Vetvicka et al., 2013; Hewlings & Kalman, 2017; Burge et al., 2019; Ching et al., 2020; Rodrigues et al., 2020; Alagawany et al., 2021).

8. Thesis objectives

Disease outbreaks raised concerns about the rapid intensification and industrialization of the aquaculture sector. To control pathogens and infectious diseases, antibiotics are usually administered. However, its use is associated with many environmental, human, and animal health concerns. On the other hand, the escalated prices of aquaculture feed ingredients, such as fishmeal and fish oil, are an obstacle to aquaculture's continuous growth. Therefore, understanding the effects of antibiotics in fish health, together with finding alternative ingredients to fishmeal and fish oil, are extremely important to maintain aquaculture sustainability and growth.

The intestine is a very complex organ, that harbours a diverse community of microorganisms. Both play an important role on nutrient digestion and absorption, and in modulation of the immune system. Therefore, understanding its biology and physiology, the effects of feed nutrients, additives, and chemicals in their structure, the microbiota dynamics, and the inflammatory process is crucial. This knowledge will help to improve fish and shrimp immunity and health, and decrease the use of antibiotics and the nutritional costs, as healthier animals are more resistant to pathogens, external factors, and inflammatory conditions.

Since the intestinal health might influence the overall performance and health of both fish and shrimp, this work intended to:

- recognize the effects of antibiotics on intestinal health and their influence at a systemic level;
- understand the impact of distant protein sources in the recovery of antibiotic therapeutics;
- evaluate the effects of alternative feed ingredients to improve fish and shrimp health, boost the immune system, and enhance disease resistance;
- understand the intestinal inflammatory processes and evaluate the effect of dietary feed additives in their control; and,
- recognise the intestinal microbiota dynamics, comprehend the dysbiosis status, and how to manipulate the bacterial population through functional feeds.

Since the intestinal health has a strong relation with overall performance and health of fish and shrimp, its manipulation will impact on animals' homeostasis, and therefore, this thesis intends to reveal some of these links.

9. References

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Chapter II

Interactive effects of dietary oxytetracycline treatment and different protein sources in the health condition of Nile tilapia (*Oreochromis niloticus*) juveniles

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Interactive effects of dietary oxytetracycline treatment and different protein sources in the health condition of Nile tilapia (*Oreochromis niloticus*) juveniles

Abstract

This study evaluated the direct effects of therapeutic dietary administration of oxytetracycline (OTC) on the health status of Nile tilapia (*Oreochromis niloticus*) juveniles, as well as the effect of different dietary protein sources in the recovery period. To evaluate the direct effects of the medicated fed, two experimental groups were established: the control group (C group), fed with a commercial-type diet; and the oxytetracycline group (O group) fed with the medicated fed (55 mg Kg^{-1} body weight day^{-1}, 10 days). Afterwards, the recovery was evaluated for 30 days. The C group remained fed with the commercial-type diet, while the O group was divided into two groups. Half was fed with the control diet (OC group) and the other half was fed with an extreme diet rich in vegetable protein sources (OE group). Several samplings were performed, firstly to evaluate the direct effects of the oxytetracycline treatment (day 10, the end of the antibiotic treatment) and then, to evaluate the recovery using distant scenarios in terms of dietary formulations (days 11, 18, 25, and 40, the end of the recovery trial). Growth, haematological profile, plasma immunological parameters, liver oxidative stress biomarkers, and intestine gene expression were evaluated. Growth was not directly affected by the dietary treatment with OTC (day 10), however, blood monocytes counts, plasma total glutathione levels and intestinal *g6pdh* and *cat* gene expression increased in the O group. On the other hand, plasma peroxidase activity and lipid peroxidation were reduced in this group. The recovery diets helped to restore the OTC effects on monocytes (day 11), plasma peroxidase (day 11), hepatic lipid peroxidation (day 11), glutathione (OC day 25, OE day 11), *g6pdh* (OC day 11, OE day 18) and *cat* (OC day 11, OE day 25), however, the extreme diet (OE group) induced an increase of haematocrit, and a decrease of white blood cells counts, thrombocytes counts, and plasma peroxidase activity. Total glutathione levels, superoxide dismutase and catalase activities were also reduced in the group fed with the extreme diet at the end of the trial, and the gene expression of *fabp2* was downregulated in the OE group. Diets were essential in the recovery of the OTC treatment; however, the extreme diet induced several modifications in tilapia's health parameters.

1. Introduction

The omnivorous species Nile tilapia (*Oreochromis niloticus*) is one of the most farmed fish produced worldwide, with a wide range of hardiness and adaptability to different rearing systems and adverse environments (Smith et al., 1999; FAO, 2005-2021; FAO, 2020). However, the increased stocking densities and stressful conditions used in high productive aquaculture practices, are reducing fish growth, inducing diseases outbreaks and fish mortality, which leads to increasingly economic losses (Björklund & Bylund, 1990; Björklund et al., 1990; Rigos & Troisi, 2005; Sekkin & Kum, 2011). *Aeromonas hydrophila*, *Vibrio anguillarum*, *Flavobacterium columnare*, and *Edwardsiella piscicida* (previous known as *Edwardsiella tarda*), are responsible for most of the bacterial diseases in Nile tilapia (FAO, 2005-2021).

Antibiotics can be used to control bacterial infections, and are frequently administrated in formulated feeds, however, their indiscriminate use is raising concerns related to public health (bacterial resistance and residues) and environment (toxicity) (Björklund & Bylund, 1990; Alderman & Hastings, 1998; Rigos & Troisi, 2005; Sekkin & Kum, 2011; Cabello et al., 2013). Oxytetracycline (OTC) is a common antibacterial agent used in aquaculture to treat bacterial systemic infections, due to its broad-spectrum bacteriostatic activity, efficacy, low cost and cost-efficacy, legal availability, and tissue diffusion (Rigos et al., 2003; Rigos & Troisi, 2005; Sekkin & Kum, 2011; Yang et al., 2020). This antibiotic is produced by *Streptomyces* spp. fungi, and limits bacteria growth, interfering with the protein synthesis by binding to the bacterial 30S ribosomal subunit of the microbial 70S ribosomes (Chopra & Roberts, 2001; Rigos et al., 2003; Rigos & Troisi, 2005; Yang et al., 2020). Usually, is administrated in formulated fed, in a dose of 50 - 100 mg kg^{-1} body weight day^{-1}, for 7-10 days (Rigos & Troisi, 2005), however, in Portugal, the recommended dose is 55 mg Kg^{-1} body weight day^{-1} for 7-10 days (DGAV, 2020).

In several fish species, oral administration of OTC is known to affect growth performance (Reda et al., 2013; El-Sayed et al., 2014; Reda et al., 2016; Julinta et al., 2019a; Julinta et al., 2019b), cause intestinal damage and microbiota dysfunction (Navarrete et al., 2008; Limbu et al., 2018), alter the haematological and immune functions (Rijkers et al., 1980; Siwicki et al., 1989; Lundén et al., 1998; Omoregie & Oyebanji, 2002; Tafalla et al., 2002; Serezli et al., 2005; Enis Yonar et al., 2011; Guardiola et al., 2012; Reda et al., 2013; Soltan et al., 2013; El-Sayed et al., 2014; Reda et al., 2016; El-Adawy et al., 2018; Yang et al., 2020), trigger oxidative stress (Enis Yonar et al., 2011; El-Adawy et al., 2018; Limbu et al., 2018; Yang et al., 2020), originate DNA damage (Botelho et al., 2015; Limbu et al., 2018) and induce bacteria resistance (Alderman & Hastings, 1998; Miranda & Zemelman, 2002).

Several works studied the effects of OTC in fish; however, rare studies investigate the importance of the diet during or after this medicated treatment. Therefore, the present study aimed to evaluate the effect of therapeutic dietary administration of oxytetracycline on the health status of Nile tilapia, as well as, to investigate the effect of the diet in the recovery process from the medicated treatment. The later was further studied in the context of both practical and extreme formulations in order to better understand the impact of extreme and cost-effective diets in the health condition of tilapia following OTC treatment.

2. Material and methods

2.1. Fish and acclimatization period

This trial followed FELASA category B and C recommendations and was conducted according to the European and Portuguese guidelines on the protection of animals used for scientific purposes “Directive 2010/63/UE” and “Decreto-Lei n. º 113/2013 de 7 de Agosto”. The trial was performed at UTAD (University of Trás-os-Montes e Alto Douro) facilities, Vila Real, Portugal.

Three hundred and fifteen juveniles of Nile tilapia from UTAD facilities, weighing 118 ± 0.8 g were randomly distributed among 9 tanks of 300 L of water capacity (35 fish each tank), in a freshwater recirculating system. A photoperiod of 10 h light 14 h dark^{-1} was applied. Water dissolved oxygen was maintained above 90 % and the water temperature was 23 ± 0.5 ºC. All animals were acclimatized to the experimental conditions, for 15 days and fed with a control diet (C group), a commercial-type diet that fulfilled the known nutritional requirements for Nile tilapia (Table 1). The diet was produced by Sparos Lda. (Olhão, Portugal) and the fish were fed twice a day, to apparent visual satiety.

Table 1: Ingredient and chemical composition of the diets.

Ingredients (% feed)*	Control diet	Oxytetracycline diet	Plant diet
Fishmeal 60	5.0	5.0	
Poultry meal 65	5.0	5.0	
Wheat gluten meal	0.5	0.5	0.5
Corn gluten meal	7.0	7.0	7.0
Soybean meal 44	22.0	22.0	22.0
Soybean meal full fat			11.7
Sunflower meal			11.7
Wheat meal	15.0	15.0	12.3
Wheat bran	9.0	9.0	9.0
Rice bran full fat	12.0	12.0	12.0
Corn meal	15.6	15.6	5.7
Soybean oil	4.6	4.6	4.0
Vitamin and mineral premix	1.0	1.0	1.0
Antioxidant	0.2	0.2	0.2
Sodium propionate	0.1	0.1	0.1
Monoammonium phosphate	1.0	1.0	1.0
L-Histidine	0.2	0.2	0.2
L-Lysine	0.8	0.8	0.8
L-Threonine	0.2	0.2	0.2
L-Tryptophan	0.2	0.2	0.2
L-Valine	0.2	0.20	0.2
DL-Methionine	0.4	0.4	0.4
Oxytetracycline		0.3	
Proximate analyses (% dry matter)			
Crude protein	29.0		29.0
Crude fat	10.0		10.0
Fiber	3.9		6.8
Starch	25.4		17.6
Ash	6.5		6.2
Gross energy	18.9		19.0

***Fishmeal 60:** CONRESA 60 - 65 % CP, 10 % CF, Conserveros Reunidos S.A., Spain; **Poultry meal 65:** 65 % CP, 12 % CF, SAVINOR UTS, Portugal; **Wheat gluten meal:** VITEN - 81 % CP, 2.1 % CF, Roquette, France; **Corn gluten meal:** 58 % CP, 4 % CF, MPS, France; **Soybean meal 44:** solvent extracted soybean meal - 43 % CP, 2.7 % CF, CARGILL, Spain; **Soybean meal full fat:** 36 % CP, 17 % CF, Ribeiro & Sousa Lda, Portugal; **Sunflower meal:** solvent extracted dehulled sunflower meal - 43 % CP, 3 % CF, MAZZOLENI SPA, Italy; **Wheat meal:** 10.2 % CP; 1.2 % CF, MOLISUR, Spain; **Wheat bran:** 14.8 % CP, 4.7 % CF, Ribeiro e Sousa Lda, Portugal; **Rice bran full fat:** 13.3 % CP; 16.3 % CF, Ribeiro e Sousa Lda, Portugal; **Corn meal:** 8.6 % CP; 4.3 % CF, Casa Lanchinha, Portugal; **Soybean oil:** J.C. Coimbra, Portugal; **Vitamin and mineral premix:** PREMIX Lda, Portugal - Vitamins (IU or mg kg^{-1} diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 500 mg; inositol, 500 mg; biotin, 3 mg; calcium pantothenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg kg^{-1} diet): copper sulfate, 9 mg; ferric sulfate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulfate,7.5 mg; sodium chloride, 400 mg; excipient wheat middling's; **Antioxidant:** VERDILOX PX, Kemin Europe NV, Belgium; **Sodium propionate:** Premix Lda, Portugal; **Monoammonium phosphate:** Windmill Aquaphos - 26 % P, Aliphos Rotterdam B.V, The Netherlands; **L-Histidine:** 99 %, Sigma-Aldrich, USA; **L-Lysine:** 99 %: Ajinomoto Eurolysine SAS, France; **L-Threonine:** 98.5 %, Evonik Nutrition & Care GmbH, Germany, **L-Tryptophan:** 98 %, Evonik Nutrition & Care GmbH, Germany; **L-Valine:** 98 %, Evonik Nutrition & Care GmbH, Germany; **DL-Methionine:** Rhodimet NP99, ADISSEO, France; **Oxytetracycline:** Aquacen Cloridrato Oxitetraciclina 1000 mg g^{-1}, Cenavisa, Spain.

2.2. Experimental design

Firstly, we evaluated the direct effects of the medicated fed. From the 9 tanks used in the acclimatization period, animals from 3 tanks remained fed with the commercial-type diet (C group), whereas the animals from the other 6 tanks shift to the medicated fed with OTC (O group) in a dose of 55 mg Kg^{-1} body weight day^{-1} (DGAV, 2020) (Figure 1). The medicated feed was formulated by Sparos, Lda. (Olhão, Portugal), applying 0.275 % of OTC by coating in the control diet. This trial lasted for 10 days. Afterward, we evaluate recovery with different diets. In this trial, the C group (3 tanks), was maintained with the same commercial-type diet, while the group fed (6 tanks) with the medicated fed (O group) was divided (Figure 1). Half of the O group (3 tanks) were fed with the control diet (OC group), while the other half of the O group (3 tanks) was fed with an extreme diet rich in vegetable ingredients (OE group) (Figure 1). This trial lasted for 30 days. Nile tilapia juveniles were fed twice a day, until visual apparent satiety.

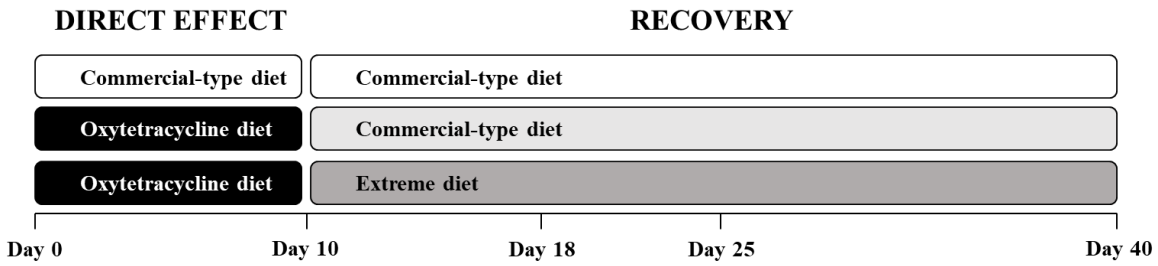

Figure 1: Experimental design.

2.3. Sample collection

Several samplings were performed. The direct effects of the OTC treatment were evaluated at day 10, the end of the antibiotic treatment. The effect of diet on the recovery was assessed several times until the end of the recovery trial, at days 11, 18, 25 and 40. Nine fish per tank were randomly selected, anesthetized by immersion in 2-phenoxyethanol (1500 ppm; Sigma), and sampled for weight, blood, and tissues. Blood was collected from the caudal vein using heparinized (2500 U.I.; Braun) syringes (23 G, 1 mL). The fish were opened, and the internal organs were placed in a petri dish. Liver samples were collected for oxidative stress and stored at -80 ºC until assayed. For gene expression, sections of 0.5 cm of the anterior and posterior intestine were collected, flushed with sodium phosphate buffer, and stored in RNAlater™ (Sigma) at -20 ºC, until assayed.

2.4. Haematological profile

The haematological profile was taken according to Machado et al. (2015), and haematocrit, total red blood cells and white blood cells counts were assessed. Haemoglobin (Spinreact kit, ref. 1001230, Spain) was also measured, according to manufacturer specifications. The mean corpuscular volume, the mean corpuscular haemoglobin and the mean corpuscular haemoglobin concentration were calculated as described by Machado et al. (2015). Blood smears were performed from homogenized blood, according to Machado et al. (2015). For the identification of neutrophils, the presence of peroxidase activity was carried out according to Afonso et al. (1998). The slides were then stained with Wright's stain (Haemacolor; Merck) to perform differential white blood cells counts in thrombocytes, lymphocytes, monocytes, and neutrophils, as described by Machado et al. (2015).

2.5. Plasma humoral immunological parameters

Total plasma protein concentration (mg mL^{-1}) was determined based on the standard curve, using a colorimetric Pierce™ BCA Protein Assay Kit (Thermo Scientific™), adapted for 96-well microplates, according to manufacturer recommendations. The anti-protease activity (%) was determined in comparison to the reference sample as described by Machado et al. (2015), however, the incubation with the phosphate buffer (NaH_2PO_4, Sigma) and azocasein (Azocasein, Sigma) was performed for 1 h, at 22 ºC, in the dark. Lysozyme activity (µg mL^{-1}) was measured using a standard curve as described by Machado et al. (2015), however, only 10 µL of plasma were incubated with 150 µL of a *Micrococcus lysodeikticus* solution (0.25 mg mL^{-1} in Na_2HPO_4, 7.098 mg mL^{-1}, pH 6.2) at 25 ºC. Total nitric oxide concentration (mg L^{-1}) was determined using a colorimetric Nitrite/Nitrate Assay Kit, (Roche Diagnostics GmbH), adapted for 96-well microplates, according to Machado et al. (2015).

Bactericidal activity was determined following the method of Machado et al. (2015) and Naghili et al. (2013), with some modifications. Briefly, *Edwardsiella piscicida* (ACC53.1, kindly provided by Prof. Alicia Toranzo) was cultured on Tryptic Soy Agar (TSA), for 24 h, at 25 ºC and then suspended in Trypticasein Soy Broth (TSB) and adjusted to OD read of ≈ 0.200 at 600 nm. This suspension was then serially diluted with TSB and cultured on TSA, to stablish colony-forming units (CFUs), using the drop plate technique with 10 µL of bacterial dilution. The bacterial suspensions correspond to 4.7 x 10^8 CFUs ml^{-1}. Plasma bactericidal activity was then determined using 20 µL of plasma suspended in 20 µL of *Edwardsiella piscicida* solution in a U-shaped 96-well plate and incubated for 2.5 h, at 25 ºC, with shaking. Saline solution 0.9 % was used instead of plasma as reference sample and 40 µL were added to another well as blank. To each well, 25 µL of 3-(4,5 dimethyl-2-

yl)-2,5-diphenyl tetrazolium bromide (1 mg mL^{-1}; Sigma) was added and incubated for 10 min, at 25 ºC, with shaking, to allow formazan formation. Plates were then centrifuged at 2000 × g for 10 min and the supernatant discarded. The precipitate was resuspended in 200 mL of dimethyl sulfoxide (DMSO; Sigma) and the OD of the dissolved formazan was measured at 560 nm. Bacterial survival was calculated comparing the samples and the positive control (maximum survival). Total bactericidal activity was expressed as the percentage of killed bacteria, calculated from the difference between the samples and the positive control (100 % living bacteria).

Total peroxidase activity (units mL^{-1}) was measured following the procedure described by Machado et al. (2015) and calculated by defining one unit of peroxidase as that which produces an absorbance change of 1 OD. All the analyses were conducted in duplicate.

2.6. Liver oxidative stress biomarkers

Liver tissue samples were homogenized (1:10 g wt v^{-1}) with K phosphate buffer (0.1 M, pH 7.4, Sigma) as described by Gravato and Guilhermino (2009). Two hundred µL of the liver homogenate were used to determine lipid peroxidation, and 4 µL of butylhydroxytoluene (BHT, 4 % in methanol, Sigma) was added to each sample, to prevent artefactual lipid oxidation, as described by Torres et al. (2002). The remaining tissue homogenate was centrifuged at 12000 rpm, for 20 min, at 4°C, to evaluate other oxidative stress biomarkers.

The colorimetric Pierce™ BCA Protein Assay Kit (Thermo Scientific™) was used to determine the total protein concentration (mg mL^{-1}), as described by Peixoto et al. (2021). Catalase activity (U mg^{-1} of protein) was determined by measuring the consumption of hydrogen peroxide (H_2O_2), as described by Clairborne (1985), adapted to the microplate reader by Almeida et al. (2010), and adjusted by Peixoto et al. (2021). The lipid peroxidation (nmol g^{-1}) was determinate with a microplate reader as adapted by Almeida et al. (2010), by measuring thiobarbituric acid-reactive substances (TBARS) as suggested by Bird and Draper (1984), with some adaptations performed by Peixoto et. al (2021). Total glutathione levels (nmol mg^{-1} of protein) were determined according to Griffith (1980), adapted to the microplate reader by Baker et al., (1990), with some modifications suggested by Peixoto et al. (2021).

Superoxide dismutase activity (U mg^{-1} of protein) was measured according to Flohe & Otting, 1984, following adaptations for microplate reader, as suggested by Lima et al. (2007), with some modifications. Briefly, concentrations of liver homogenates were adjusted to 0.3 mg mL^{-1} of protein concentration, with K-phosphate buffer (100 mmol L^{-1}, pH 7.4) and 50 µL of these suspensions were added to flat-bottomed 96-well plates. K-phosphate buffer

(100 mmol L^{-1}, pH 7.4) was used instead of liver homogenate as a blank. Then, 200 µL of the reaction solution (0.064 mmol L^{-1} of xanthine in 1 mmol L^{-1} of NaOH and 0.0273 mmol L^{-1} cytochrome c in 50 mmol L^{-1} Na - phosphate buffer (pH 7,8) with 1 mmol L^{-1} Na-EDTA) and 50 µL of xanthine oxidase solution (0.03 U mL^{-1}) were added to each well. Final assay concentrations per well were 0.042 mmol L^{-1} of xanthine, 0.018 mmol L^{-1} cytochrome c, 0.005 U mL^{-1} xanthine oxidase solution, 0.06 mmol L^{-1} of NaOH, 30 mmol L^{-1} of NaH_2PO_4 and Na_2HPO_4 and 0.62 mmol L^{-1} Na-EDTA. The reaction was monitored by the formation of superoxide anion at 550 nm every 20 sec for 3 min on Synergy HT microplate reader. Enzyme activity was expressed as enzyme units per millilitre of total protein (U mL^{-1} protein). All the analyses were conducted in triplicates.

2.7. Intestinal gene expression

Anterior and posterior portions of the intestine were analysed for mRNA expressions to evaluate innate immunity, oxidative stress, inflammation, and DNA damage. Around 30 mg of tilapia's intestine were placed in a 2 mL tube containing 0.5 mL of Trizol (Nzol Reagent, NZYTech, Portugal) and 2 Zirconium Oxide-coated ceramic grinding spheres (2 mm diameter) and homogenised in a Precellys 24 tissues homogenizer (Bertin Ins., France) by 2 cycles of 6000 x g for 20 seconds. After that, 150 µl of chloroform was added to the homogenate, gently vortexed and centrifuged for 15 min at 12000 x g and 4 ºC. Around 300 µl of the aqueous phase were transferred to a new 1.5 ml tube with 300 µl of 70 % ethanol and gently homogenised. After this step, the RNA extraction was done with a NZY Total RNA Isolation Kit (NZYTech, Lisbon, Portugal) following the manufacturer's specifications. Quantification and purity of RNA were assessed by spectrophotometry and the 260:280 and 260:230 ratios were defined in a DeNovix DS 11 FX Instrument (DeNovix, US). RNA integrity was verified through 1.5 % gel electrophoresis. First-strand cDNA was synthesized with NZY First-Strand cDNA Synthesis Kit (NZYTech, Lisbon, Portugal) following the manufacturer's specifications. Primers were designed using NCBI Primer Blast Tool (Table 2). Related genes with the redox system (*g6pdh*, *cat*, *sod2*), pro-inflammatory (*tnfα* and *il1β*) and anti-inflammatory cytokines (*tgfβ* and *il10*), programmed cell death (*casp3*), immunoglobulins (*igm*), DNA repair (*pcna*), and metabolism (*fabp2* and *aqp10*) were tested.

Table 2: Oligonucleotide primers used in this study for Real-time PCR analysis of gene expression.

Target gene name	Symbol	Accession nr.	Forward primer (5´-3´) Reverse primer (5´-3´)	PL[1]	E[2]A P
Glucose-6-phosphate 1-dehydrogenase	*g6pdh*	XM_005478106.4	ACAGGAACTGTCAGCCCACCTT AGCACCATGAGGTTCTGGACCA	102	2.02 1.96
Catalase	*cat*	XM_019361816.2	ATGAGGAGGAGCGACAGAGACT ACAGCCTTCAAATTCTCGACCAT	100	1.95 1.99
Superoxide dismutase 2	*sod2*	XM_003449940.5	TTGCAGGGGACAACAGGTCT GACGCTCACTCACGTTCTCC	142	2.06 1.94
Tumour necrosis factor alpha	*tnfα*	NM_001279533.1	GCTGGAGGCCAATAAAATCA CCTTCGTCAGTCTCCAGCTC	129	1.86 2.24
Interleukin 1 beta	*il1β*	XM_005457887.1	TGGTGACTCTCCTGGTCTGA GCACAACTTTATCGGCTTCCA	86	2.00 1.99
Transforming growth factor beta	*tgfβ*	XM_003459454.2	GTTTGAACTTCGGCGGTACTG TCCTGCTCATAGTCCCAGAGA	80	1.86 2.02
Interleukin 10	*il10*	XM_003441366.2	CTGCTAGATCAGTCCGTCGAA GCAGAACCGTGTCCAGGTAA	94	1.97 1.91
Caspase-3	*casp3*	GQ421464.1	GGCTCTTCGTCTGCTTCTGT GGGAAATCGAGGCGGTATCT	80	1.91 1.95
Proliferating cell nuclear antigen	*pcna*	XM_003451046.2	CCCTGGTGGTGGAGTACAAG AGAAGCCTCCTCATCGATCTTC	80	1.86 2.01
Immunoglobulin M heavy chain	*igm*	KC677037.1	GGCTCCTACAATGAACCATCCA ATGTTTGACCCCCAGTACCAA	83	1.95 1.99
Fatty acid binding protein 2	*fabp2*	XM_003452249.4	AGCAGCACTTTCCGAAGCAT CAAGGTCCACGCACCTGATAG	99	2.07 1.93
Aquaporin 10	*aqp10*	XM_003452451.5	TCTGGGTTTTGCTCTGGGAG CGGGTTTAAATGAGCACCCGA	73	2.10 1.96

[1] Product length or amplicon (nt)

[2] Efficiency of PCR reactions of the anterior (A) and posterior (P) intestine

The efficiencies of the primer pairs for the genes of interest were obtained using serial five-fold dilutions of cDNA by calculating the slope of the regression line of the cycle thresholds (Ct) versus the relative concentration of cDNA as described by Machado et al. (2018). Melting curve analyses were also performed to verify the presence of primer dimers. Quantitative PCR assays were performed with an iQ5 Real-Time PCR Detection System (BioRad) using 4.4 µl of diluted cDNA (1:15 dilution) mixed with 5 µl of iQ SYBR green 2x Supermix (Bio-Rad) and 0.3 µl (10 mM) of each specific primer in a final volume of reaction of 10 µL. The standard cycling conditions were, initially one cycle of 95 ºC for 10 min, one cycle of 95 ºC for 15 sec and one cycle of 60ºC for 1 min, followed by 40 cycles of 95 ºC for 15 sec, one cycle of 95 ºC for 1 min and one cycle of 60 ºC for 30 sec, followed by a melting curve from 60 ºC to 95 ºC, with increments of 0.5 ºC for each 0.5 sec, and finally a cycle of 95 ºC for 15 sec. All reactions were carried out as technical duplicates. The expression of the target gene was normalized using the expression of the elongation factor 1-alpha (*ef1α*) as reference gene.

2.8. Statistical analysis

All results were expressed as means and standard deviation. Data were analysed for normality and homogeneity of variance and, when necessary, log transformed before being treated statistically, using one-way ANOVA followed by Tukey post hoc test to identify differences in the experimental treatments. Statistical analysis was performed using IBM SPSS Statistics 24. The level of significance used was $p \leq 0.05$ for all statistical tests.

3. Results

3.1. Growth performance and Survival rate

The feeding behaviour of the juvenile tilapias was normal, and the entire feed rations were consumed. There were no mortalities associated with the experimental dietary treatments. As seen in Figure 2, final body weight was not affected directly by the dietary treatment with OTC (O group, day 10). However, the OE group presented a significant decrease in the final body weight at the end of the recovery trial (day 40).

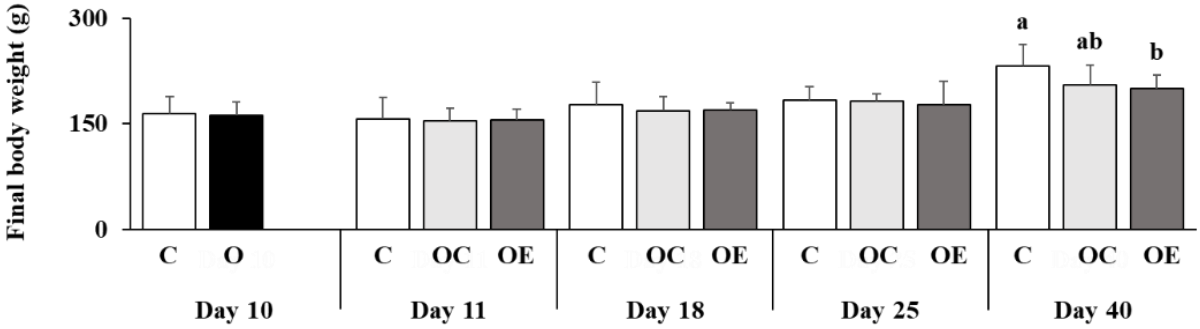

Figure 2: Final body weight of Nile tilapia fed with OTC medicated feed for 10 days. Evaluation of the effects at day 10 (direct effect), and at days 11, 18, 25, 40 (recovery). **C** (control diet), **O** (OTC diet), **OC** (OTC + control diet), **OE** (OTC + extreme diet). Different letters mean significant differences among the dietary treatments ($p < 0.05$).

3.2. Haematological profile

The red blood cell profile (Figure 3) did not show any statistical change after the dietary OTC treatment (O group, day 10). Nevertheless, the OC group presented an increase of haematocrit on day 40, and the OE group showed an increase of haematocrit on days 11 and 40, an increase of red blood cells counts on days 11 and 18, as well as an improved mean corpuscular volume on day 40.

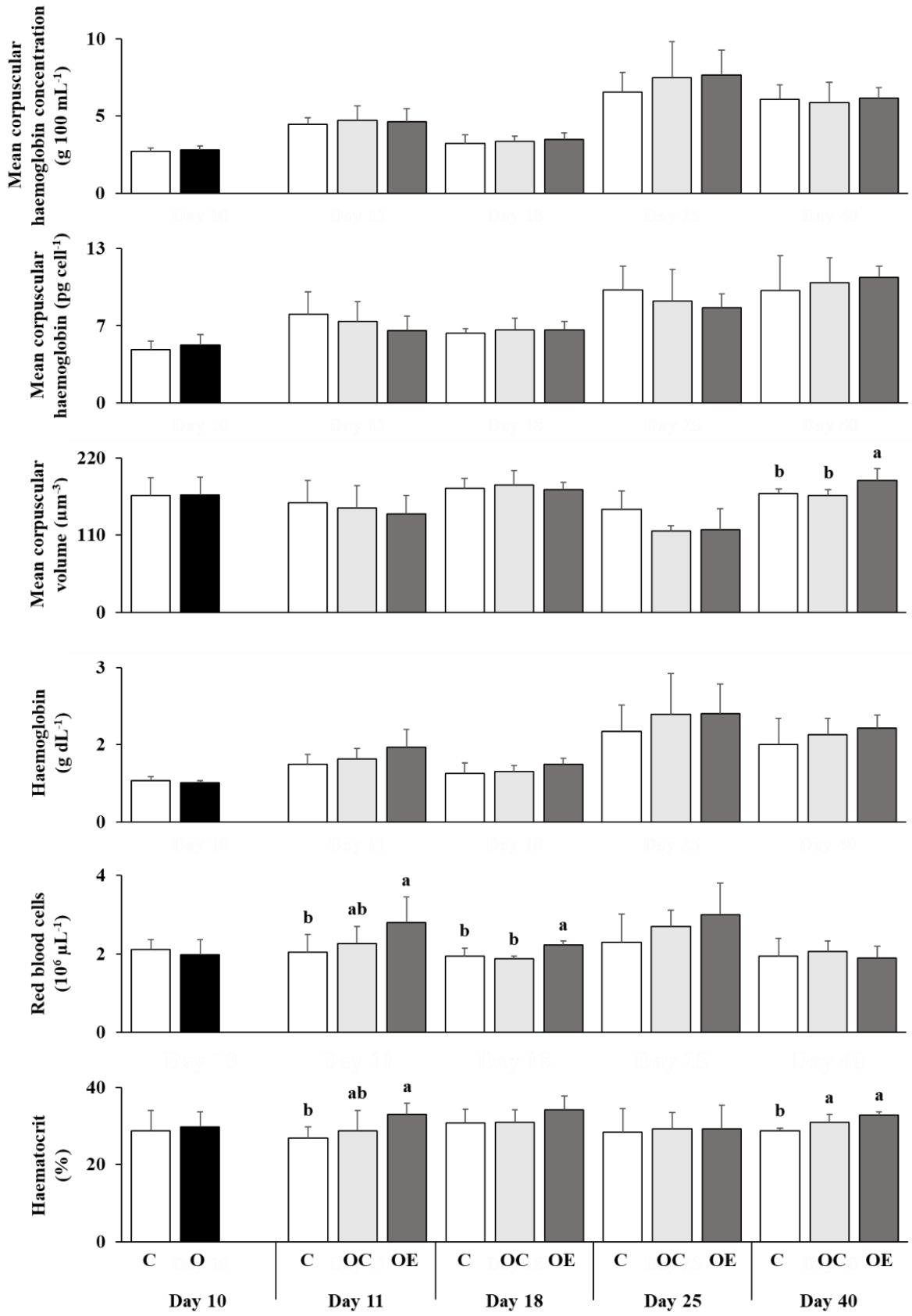

Figure 3: Red blood profile of Nile tilapia fed with OTC medicated feed for 10 days. Evaluation of the effects at day 10 (direct effect), and at days 11, 18, 25, 40 (recovery). **C** (control diet), **O** (OTC diet), **OC** (OTC + control diet), **OE** (OTC + extreme diet). Different letters mean significant differences among the dietary treatments ($p < 0.05$).

Monocytes increased significantly after the dietary treatment with OTC (O group, day 10), but they returned to normal values on day 11, with both the recovery diets (OC and OE groups) (Figure 4). The OC group presented a decrease of thrombocytes on day 40, and the OE group showed a decreased of white blood cells and thrombocytes on day 40.

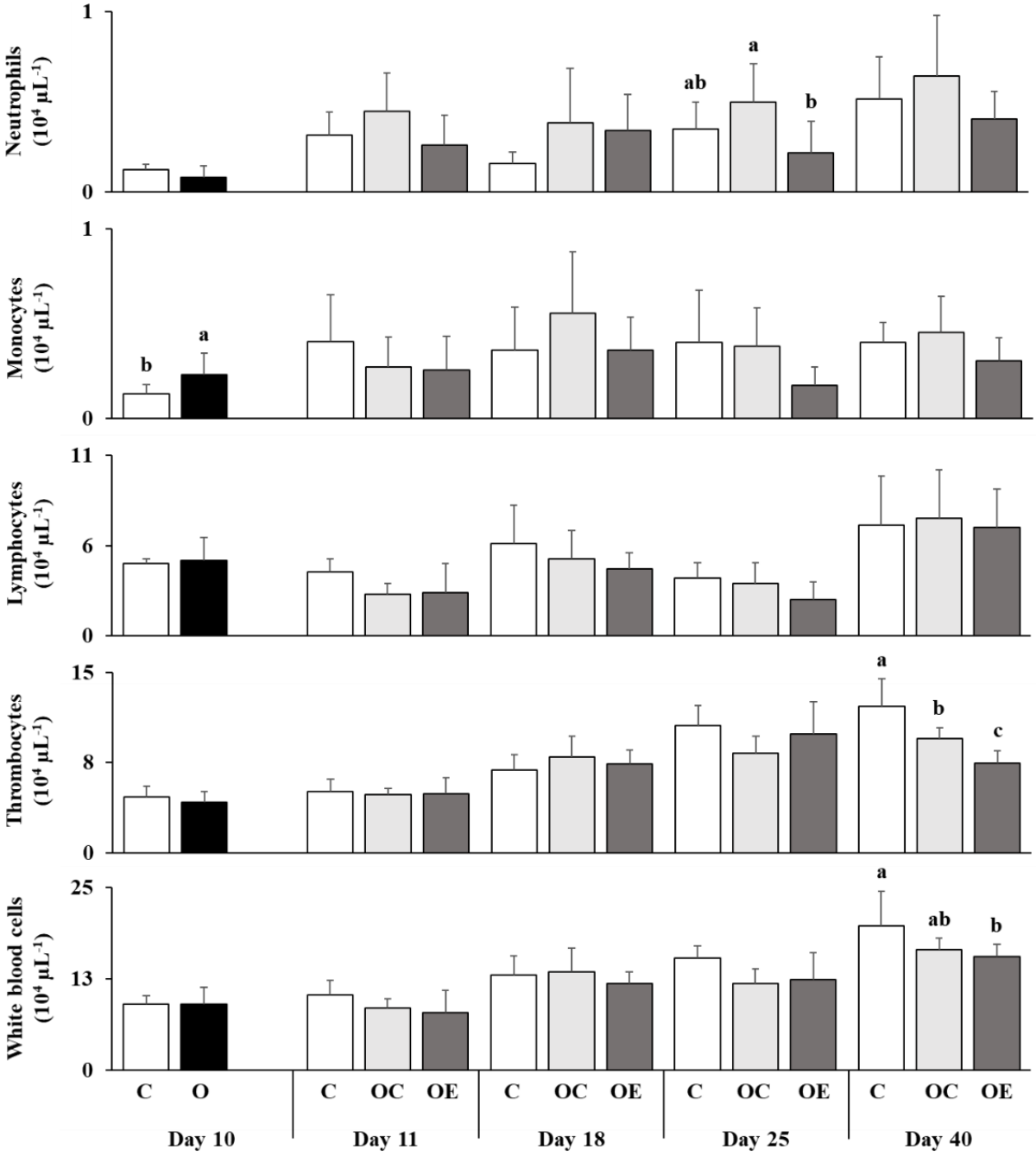

Figure 4: White blood profile of Nile tilapia fed with OTC medicated feed for 10 days. Evaluation of the effects at day 10 (direct effect), and at days 11, 18, 25, 40 (recovery). **C** (control diet), **O** (OTC diet), **OC** (OTC + control diet), **OE** (OTC + extreme diet). Different letters mean significant differences among the dietary treatments ($p < 0.05$).

3.3. Plasma humoral immunological parameters

Peroxidase activity decreased in the O group at day 10, but the recovery happened on day 11 with both the recovery diets (OC and OE groups) (Figure 5). The OC group presented a decrease of anti-protease activity of plasma on days 11 and 18, while the OE group, presented a decrease of anti-protease activity on days 11 and 18, as well as a decrease of peroxidase activity on days 25 and 40.

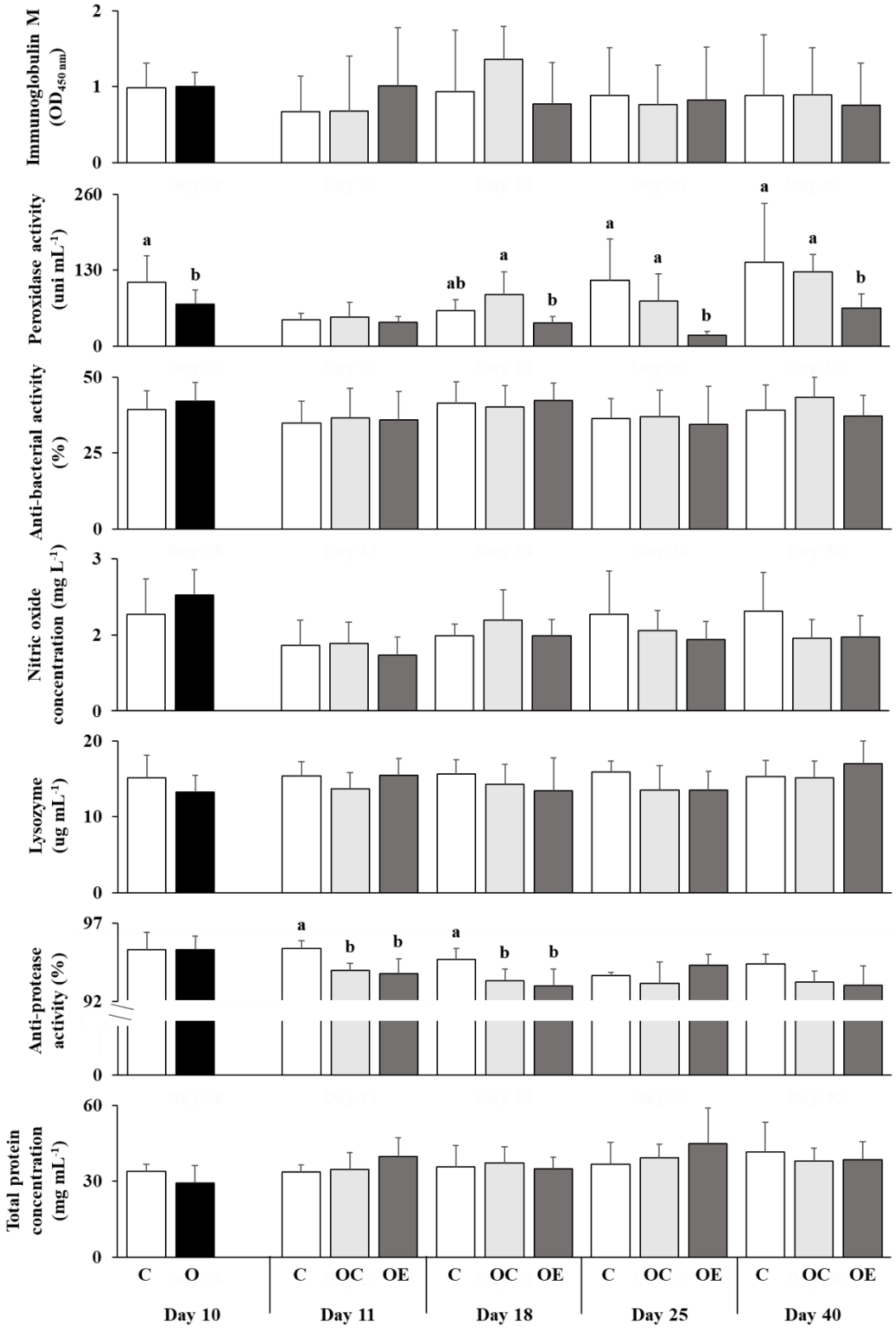

Figure 5: Humoral immunological parameters of Nile tilapia fed with OTC medicated feed for 10 days. Evaluation of the effects at day 10 (direct effect), and at days 11, 18, 25, 40 (recovery). **C** (control diet), **O** (OTC diet), **OC** (OTC + control diet), **OE** (OTC + extreme diet). Different letters mean significant differences among the dietary treatments ($p < 0.05$).

3.4. Liver oxidative stress biomarkers

As Figure 6 shows, total glutathione increased in the O group at day 10, however, the recovery happened on day 11 for the OE group and on day 25 for the OC group. On the other hand, lipid peroxidation TBARS levels decreased in the O group at day 10, but they returned to normal values on day 11, with both recovery diets (OC and OE groups). The OC group presented an increase of catalase and superoxide dismutase activities, as well as total glutathione levels on day 11, while having a decrease of total glutathione and lipid peroxidation TBARS levels on day 18. Also, the OC group presented a decrease of superoxide dismutase activity and total glutathione levels on day 40. On the other hand, the OE group showed a decrease of total glutathione levels on day 18, and a decrease of catalase and superoxide dismutase activities, as well as total glutathione levels on day 40.

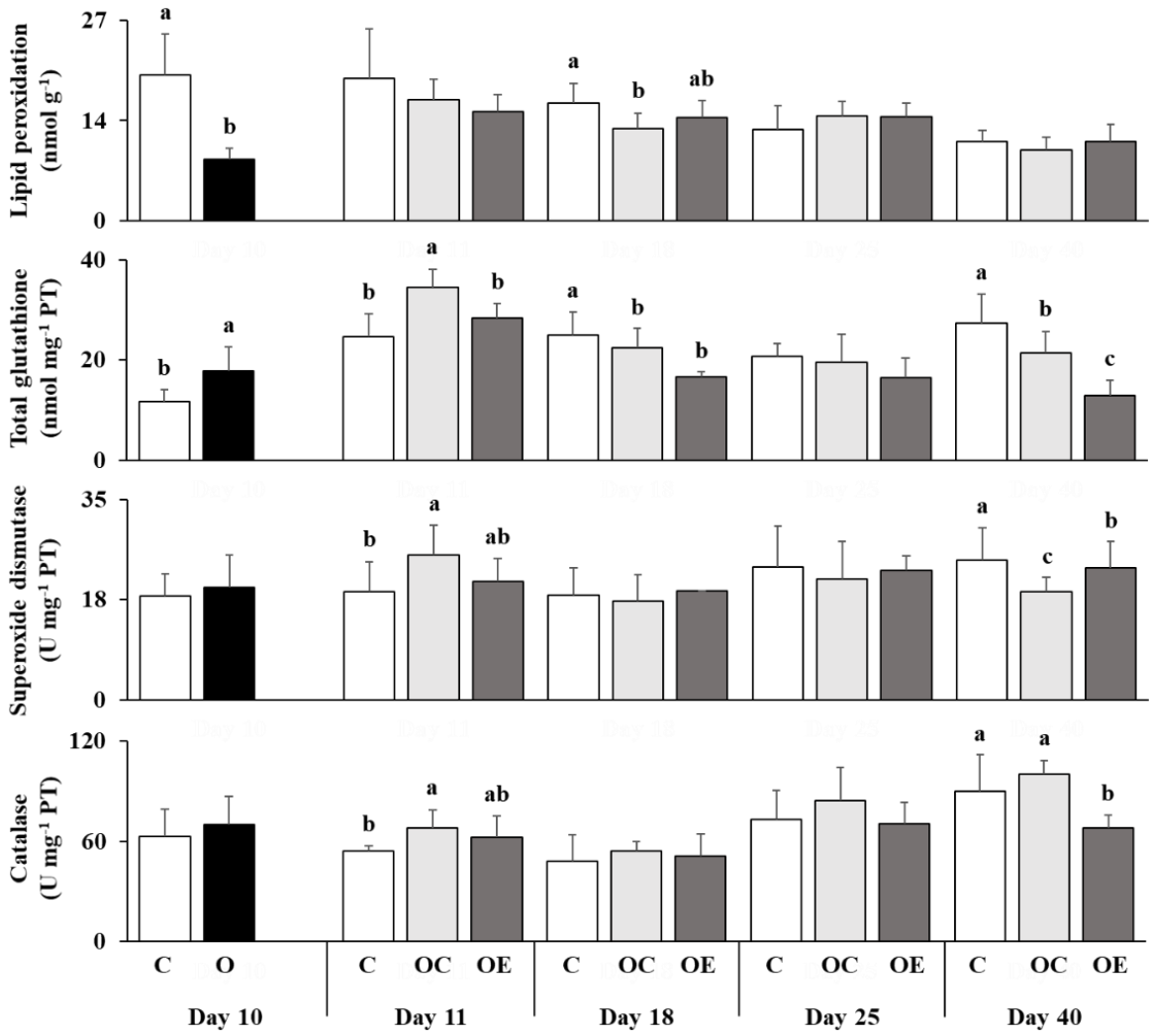

Figure 6: Oxidative stress biomarkers in the liver of Nile tilapia fed with OTC medicated feed for 10 days. Evaluation of the effects at day 10 (direct effect), and at days 11, 18, 25, 40 (recovery). **C** (control diet), **O** (OTC diet), **OC** (OTC + control diet), **OE** (OTC + extreme diet). PT (total protein concentration). Different letters mean significant differences among the dietary treatments ($p < 0.05$).

3.5. Intestinal gene expression

The gene expression profile in the anterior and posterior intestine of Nile tilapia after the OTC treatment is described in Figure 7. Though several genes weren´t directly affected by the OTC medicated feed (O group) on day 10, the oxidative stress-related genes (*g6pdh* and *cat*) were upregulated in the anterior intestine, while the immunoglobulin (*igm*) and the metabolic-related gene (*fabp2*) were downregulated in the posterior intestine. The recovery of the OC group was faster (day 11) for all the genes affected by the OTC treatment, while the OE group recover on day 11 for the *igm* and *fabp2* genes, on day 18 for the *g6pdh*, and on day 25 for the *cat* gene.

In the anterior intestine, the OC group did not present any change on days 11 and 18; however, on day 25, *igm* decreased; and on day 40 *tnf*α, *il1*β, *tgf*β, and *igm* decreased in this group. In the posterior intestine, on day 11, *sod* decreased and *aqp10* increased; on day 18, there were no changing on the gene expression; on day 25, *sod* decreased; and on day 40, *fabp2* decreased.

The OE group, in the anterior intestine, presented on day 11, a decrease of *tgf*β and *il10*; on day 18, an increase of *sod*, *il1*β, *fabp2*, and *aqp10*; on day 25, a decrease of *igm*; and on day 40, there were no changes in the gene expression. In the posterior intestine, on day 11, *cat* and *aqp10* increased, and *sod* decreased; on day 18, *cat*, *tnf*α, *il1*β, *il10*, *pcna*, *fabp2*, and *aqp10* increased; on day 25, *cat* and *il1*β increased; and on day 40, *fabp2* decreased.

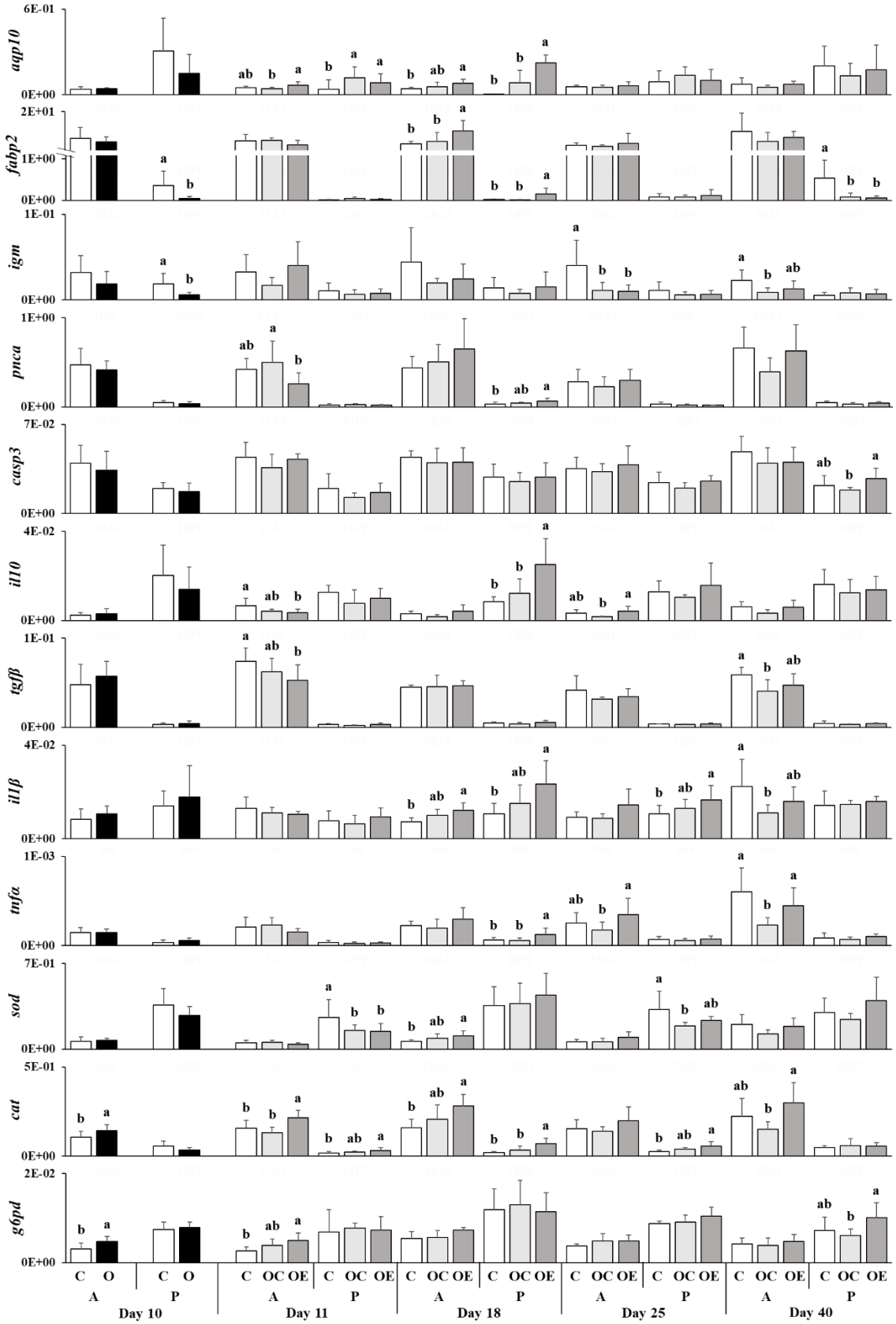

Figure 7: Normalized gene expression profile in the intestine of Nile tilapia fed with OTC medicated feed for 10 days. Evaluation of the effects at day 10 (direct effect), and at days 11, 18, 25, 40 (recovery). **C** (control diet), **O** (OTC diet), **OC** (OTC + control diet), **OE** (OTC + extreme diet). A (anterior intestine), P (posterior intestine). Different letters mean significant differences among the dietary treatments ($p < 0.05$).

4. Discussion

High stocking densities used in intensive aquaculture of Nile tilapia, as well as other chronic stressful conditions, provide an ideal atmosphere for diseases outbreaks (Björklund & Bylund, 1990; Björklund et al., 1990; Rigos & Troisi, 2005; Sekkin & Kum, 2011). Antibiotics can be used to treat diseases, however, OTC dietary treatments can affect growth performance (Reda et al., 2013; El-Sayed et al., 2014; Reda et al., 2016; Julinta et al., 2019a; Julinta et al., 2019b), cause intestinal damage and microbiota dysfunction (Limbu et al., 2018), alter the haematological and immune functions (Omoregie & Oyebanji, 2002; Reda et al., 2013; Soltan et al., 2013; El-Sayed et al., 2014; Reda et al., 2016; El-Adawy et al., 2018), trigger oxidative stress (El-Adawy et al., 2018; Limbu et al., 2018), and origin DNA damage (Botelho et al., 2015; Limbu et al., 2018) in Nile tilapia.

4.1. Oxytetracycline direct effects

Growth performance and survival are important factors for fish farmers (Limbu et al., 2018). In this study there was no mortality associated with the experimental diets, however, other researchers found reduced survival rates in tilapia fed with other concentrations of OTC (Julinta et al., 2019a, 240 mg kg^{-1} biomass day^{-1}, 30 days). In the present study, growth was not directly affected by the dietary treatment with OTC, in line with previous studies. For instance, no differences in weight gain were also found by Limbu et al. (2018) after a dietary treatment with OTC with a 2 g kg^{-1} diet (80 mg kg^{-1} body weight day^{-1}) for 84 days. However, Julinta et al. (2019a) (240 mg kg^{-1} biomass day^{-1}, 30 days) and Julinta et al. (2019b) (800 mg kg^{-1} biomass day^{-1}, 10 days) described a progressive reduction of the biomass with the increase of OTC concentration. As OTC may affect pellet palatability, reducing feed intake (Toften et al., 1995; Toften and Jobling, 1997), it is expected that fish, especially diseased fish with reduced appetite and diminished ability to absorb and metabolize drugs, can exhibit lower values of growth after medicated treatments (Björklund & Bylund, 1990; Björklund et al., 1990; Rigos & Troisi, 2005).On the other hand, Reda et al. (2013) (100 mg kg^{-1} diet, 12 weeks), El-Sayed et al. (2014) (1g kg^{-1} diet, for 4 or 8 weeks), and Reda et al. (2016) (0.5 g kg^{-1} diet, 60 days), found an increase of final body weight or weight gain of Nile tilapia after OTC medicated treatments. For several years, antibiotics were used as growth promoters, but their indiscriminate use, with its toxicity and bacterial resistance, lead to strictly legislations, banning the use of antibiotics as feed additives to stimulate growth in many European countries (Chopra & Roberts, 2001; Rigos & Troisi, 2005; Cabello et al., 2013; Reda et al., 2013).

Despite the low bioavailability when administrated orally, due to decreased membrane permeability induced by its lipophilicity and complex formation with cations in the water or

feed, OTC is well absorbed in the intestine, reaching elevated concentrations rapidly in the blood (Cravedi et al., 1987; Björklund & Bylund, 1990; Rigos et al., 2003; Rigos & Troisi, 2005; Enis Yonar et al., 2011; Sekkin & Kum, 2011), and blood haematological parameters can be an important tool to monitor health and stress responses (Fazio, 2019). Results form the present study revealed that the dietary treatment with OTC did not produce any significant alterations in the haematological profile, however, monocytes increased after the dietary treatment with OTC. Soltan et al. (2013) found an increase of red blood cells and haemoglobin in tilapia, after a dietary treatment with OTC (80 mg kg^{-1} diet, 80 days). The increase of thrombocytes was also found by El-Adawy et al. (2018) (500 mg kg^{-1} diet, 2 weeks) and Reda et al., 2016 (0.5 g kg^{-1} diet for 60 days) in tilapia. On the other hand, a diminution of red blood cells (Omoregie & Oyebanji, 2002, 2.5 % diet for 1 week or 1.25 % diet for 2 weeks), haematocrit (Omoregie & Oyebanji, 2002, 1.25 % diet for 1 week; Reda et al., 2016, 0.5 g kg^{-1} diet for 60 days), haemoglobin (Omoregie & Oyebanji, 2002, 2.5 % diet for 1 week or 1.25 % diet for 2 weeks), white blood cells (Omoregie & Oyebanji, 2002, 0.63 % diet for 1 week; El-Sayed et al., 2014, 1g kg^{-1} diet, 4 or 8 weeks) and thrombocytes (Omoregie & Oyebanji, 2002, 5 % diet for 1 week or 0.63 % diet for 2 weeks) have also been described in Nile tilapia fed with OTC.

The contradictory effects of the dietary treatment with OTC in Nile tilapia seem to be related to the concentration and duration of the treatment (Omoregie & Oyebanji, 2002; Reda et al., 2013; Soltan et al., 2013; El-Sayed et al., 2014; Botelho et al., 2015; Reda et al., 2016; El-Adawy et al., 2018; Limbu et al., 2018; Julinta et al., 2019a; Julinta et al., 2019b), however, fish species, culture conditions, antibiotic dose, or route of administration can also influence the outcomes derived from the oxytetracycline treatments in fish (Grondel et al., 1987; Björklund & Bylund, 1990; Björklund et al., 1990; Yang et al., 2020).

OTC is easily distributed throughout the body, the tissue penetration is significant, and the elimination is slow, persisting in several fish tissues, such as liver, muscle, and kidney (Cravedi et al., 1987; Björklund & Bylund, 1990; Rigos et al., 2003; Rigos & Troisi, 2005; Enis Yonar et al., 2011; Sekkin & Kum, 2011). As the kidney is an important immune and hematopoietic organ in fish, the presence of OTC in this organ, may influence the immune cells and the immune responses (Grondel et al., 1987; Enis Yonar et al., 2011). In this study, only plasma peroxidase activity decreased after the dietary treatment with OTC, but there was also a slight decrease of lysozyme activity. Reda et al. (2016) (0.5 g kg^{-1} diet, 30 days) and El-Adawy et al. (2018) (500 mg kg^{-1} diet, 2 weeks) found decreased lysozyme activity in plasma in fish fed with OTC. A decrease of protease activity was also verified by Limbu et al., 2018, after dietary treatments with OTC (80 mg kg^{-1} body weight day^{-1}, 84 days). On contrary, Reda et al. (2013) (100 mg kg^{-1} diet, 12 weeks) and El-Sayed et al. (2014) (1 g kg^{-1} diet, 4 weeks), verified an increase of lysozyme activity in tilapia fed with OTC.On the other

hand, nitric oxide concentration and bactericidal activity seem to be improved non-significantly after the OTC treatment, in the present study. El-Sayed et al. (2014) described an increase in serum bactericidal activity after fish were fed with OTC (1 g kg^{-1} diet for 8 weeks), however, Reda et al. (2016) found a decrease in nitric oxide concentration in animals fed with OTC (0.5 g kg^{-1} diet, 30 days). Once again, concentration and duration of the treatment seem to be important aspects when evaluating OTC effects.

Antibiotics can induce adverse effects in several tissues. The liver, as the major organ of detoxification, is one of the most affected (Guillouzo & Guguen-Guillouzo, 2020). Specialized enzymes, such as superoxide dismutase, catalase, glutathione peroxidase, glutathione s-transferase, and glutathione reductase, as well as non-enzymatic components, such as glutathione, are the primary defence mechanism against oxidative stress (Enis Yonar et al., 2011; Biller & Takahashi, 2018; Saikia & Chowdhury, 2020). The oxidative responses are variable due to different rates of free radical generation, susceptibility to oxidative damage, and antioxidant capacities of the tissues (Enis Yonar et al., 2011; Biller & Takahashi, 2018). Antibiotic treatments in fish normally induce oxidative stress in the liver, especially with a long-lasting therapy (Tafalla et al., 1999; El-Adawy et al., 2018; Limbu et al., 2018). Lipid peroxidation is a valuable indicator of oxidative damage of cellular components, as oxygen and nitrogen reactive species attack the cell membranes, and induce lipid peroxidation (Enis Yonar et al., 2011). In this study, despite the increase in total glutathione in the group fed with OTC, no lipid peroxidation occurred. The antioxidant defence mechanisms seem to be effective and there was no oxidative degradation of lipids at the recommended dose and time. However, other studies indicate the suppression of superoxide dismutase (El-Adawy et al., 2018, 500 mg kg^{-1} diet, 2 weeks) and glutathione peroxidase (El-Adawy et al., 2018, 500 mg kg^{-1} diet, 2 weeks), as well as an increase of lipid peroxidation (El-Adawy et al., 2018, 500 mg kg-1 diet, 2 weeks; Limbu et al. (2018), 80 mg kg-1 body weight day-1, 84 days) in Nile tilapia fed with OTC. Thought with non-significant difference in our work, the superoxide dismutase activity increased with the OTC treatment, as found by Limbu et al. (2018) in tilapia fed with OTC (80 mg kg^{-1} body weight day^{-1}, 84 days).

Intestines are a very important organ for the digestion and immunity of fish (Salinas & Parra, 2015). After the ingestion of OTC (day 10), the oxidative-stress related genes (*g6pdh*, *cat*) were upregulated in the anterior intestine, while *igm* and *fabp2* were downregulated. Antibiotics can induce intestinal damage, by releasing oxygen and nitrogen reactive species (Guillouzo & Guguen-Guillouzo, 2020). In *Tilapia sparrmanii*, the size of absorptive cells diminishes from the proximal intestine towards the distal, which may explain the more pronounced oxidative stress damage presented in the anterior part of the intestine than in the posterior (Okuthe & Bhomela, 2020). Thought not verified in the present study, an

upregulation of *sod* (Limbu et al., 2018, 80 mg kg^{-1} body weight day^{-1}, 84 days), *il1β* (Limbu et al., 2018, 80 mg kg^{-1} body weight day^{-1}, 84 days; Reda et al., 2016, 0.5 g kg^{-1} diet, 30 days), *tnfα* (Limbu et al., 2018, 80 mg kg^{-1} body weight day^{-1}, 84 days; Reda et al., 2016, 0.5 g kg^{-1} diet, 30 days), and *il8* (Limbu et al., 2018, 80 mg kg^{-1} body weight day^{-1}, 84 days) and downregulation of *il10* (Limbu et al., 2018, 80 mg kg^{-1} body weight day^{-1}, 84 days), were described after dietary treatments with OTC in tilapia. The OTC concentration from the present study was lower than the one used in other studies, which may explain the absence of more pronounced changes in the gene expression profile related with oxidative stress.

Immunoglobulin M is the main immunoglobulin in fish, which plays an important role in the fish immune response (Salinas & Parra, 2015), however, OTC can suppress this immunoglobulin in tilapia (Reda et al., 2013). Indeed, in this study, *igm* was significantly downregulated in the posterior intestine. In contrary to our findings, El-Adawy et al. (2018), found an upregulation of *igm* in the head kidney, as well as an increase of plasma concentration of immunoglobulin M in tilapia fed with OTC (500 mg kg^{-1} diet, 2 weeks). OTC has also been associated with cellular DNA damage (Botelho et al., 2015), however, we didn't observe any differences in the DNA repair gene, *pcna*, at day 10. Although OTC had no significant effect on the total lipid content and total fatty acids in rainbow trout, it significantly enhanced the digestibility of some unsaturated fatty acids (Cravedi et al. 1987). In our study, *fabp2*, an intracellular protein that participates in the metabolism and transport of fatty acids, was downregulated in the O group, impairing this metabolism. Nevertheless, the recommended dose of OTC in Portugal seems to be relatively safe to be used, however, concentrations above the permissible limits may be toxic to the fish (Julinta et al., 2019a).

4.2. Dietary recovery

There are only a few studies where the use of specific diets together with OTC have been assessed. High fat diets used concurrently with OTC seem to worsen the adverse effects caused by OTC dietary treatments in Nile tilapia, while high carbohydrate diets seem to attenuate the OTC health effects (Limbu, et al., 2019; Limbu, et al., 2020). In this study, we evaluate the use of different diets as a recovery tool to medicated treatments with OTC, exploring the use of a control diet, with fish meal, that fulfilled the known nutritional requirements for Nile tilapia, and the use of an extreme diet, more aggressive, with the absence of fish meal and the presence of various vegetable ingredients.

In this study, only a few adverse effects of OTC were verified, with the increase of blood monocytes counts, decrease of plasma peroxidase activity, increased total glutathione levels and decreased lipid peroxidation TBARS levels, as well as an upregulation of *g6pdh* and *cat* genes, and a downregulation of *igm* and *fabp2* genes. These results showed the

oxidative-stress, immunologic, and metabolic adverse effects that OTC can induce in Nile tilapia. However, the recovery diets were able to neutralize all these OTC effects, rapidly returning monocytes, peroxidase, lipid peroxidation TBARS levels, *igm* and *fabp2* within the control values. Also, the control diet (OC group) seemed to be faster to neutralize *g6pd* and *cat* gene expression, while the extreme diet (OE group) allowed a quicker response to decrease glutathione levels. The presence of anti-nutritional factors in extreme vegetable diets may be responsible for the recover delay of *g6pd* and *cat* gene expression, once they induce intestinal damage and inflammation (Estruch et al., 2018; Kumar et al., 2020). Inflammation and oxidative stress are pathological processes known to be related, as inflammatory cells release a high number of oxygen and nitrogen reactive species (ROS and NOS) in the inflammation site, which leads to oxidative stress, and the release of ROS and NOS, and further intestinal inflammation, results in intestinal ischemia and necrosis, creating a vicious cycle (Hewlings & Kalman, 2017; Burge et al., 2019).

As OTC can reach and persist in several tissues (Cravedi et al., 1987; Björklund & Bylund, 1990; Rigos et al., 2003; Rigos & Troisi, 2005; Enis Yonar et al., 2011; Sekkin & Kum, 2011), it is important to define its concentration in different organs with time, to better understand its effects. Paschoal et al. (2012) verified OTC concentrations in fish below the limit of quantitation after 8 days post-treatment and withdrawal periods of 6 days. On the other hand, Reda et al. (2013) found withdrawal periods of 15 days, and no residues of OTC were found by Soltan et al. (2013) after 21 days of the end of the treatment. However, Julinta et al. (2019b) describes that some health biomarkers didn't recover, even after 42 days of the cessation of the treatment.

Nevertheless, the extreme diet (OE group) induced several fluctuations in the health-related parameters analysed in this study, throughout the recovery trial, decreasing growth, disturbing the haematological profile, altering immunological parameters, affecting the oxidative stress biomarkers, and influencing the gene expression profile. Regardless of the fact that Nile tilapia is an omnivorous species accustomed to plant ingredients (Smith et al., 1999; Kuli Khan et al., 2013), some adverse effects have been associated with extreme diets (Kuli Khan et al., 2013; Hassaan et al., 2018). Normally, these types of diets are associated with poor growth and weakening of the immune system of fish (Kuli Khan et al., 2013; Ogello et al., 2017; Hassaan et al., 2018). Thought plant-based diets are sustainable and cheaper alternatives to fish meal, the presence of anti-nutritional factors, high amounts of fiber, and imbalanced amino acid profiles, can reduce palatability, digestibility, and bioavailability of nutrients, can impair the complete replacement of fish meal by a vegetable meal (Shiau et al., 1990; Al-Kenawy et al., 2008; Kuli Khan et al., 2013; Ogello et al., 2014; Ogello et al., 2017; Hassaan et al., 2018).

5. Conclusions

The present results indicate that the recommended dose of OTC in Portugal is relatively safe to be used in Nile tilapia, however, this dosage can influence the physiological status of the fish. Also, the diet is an extremely important element in the process of recovery from medicated treatments, as an equilibrated diet provides a faster and stronger response, and a poor diet delays the recovery and induces several modifications in the health-related parameters. These findings can be very useful for researchers and aquaculture in the evaluation of the health status of Nile tilapia after the oxytetracycline administration.

6. Acknowledgements

We thank Professor Alicia Toranzo for kindly providing the bacterial strain (*Edwardsiella piscicida*, ACC53.1) used in this study. C. Teixeira was supported by SPAROS and FCT - Foundation for Science and Technology, Portugal, through grant PD/BDE/135541/2018.

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Chapter III

Modulation of intestinal morphology and microbiota diversity by the dietary administration of oxytetracycline in Nile tilapia (*Oreochromis niloticus*)

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Abstract

The effects of the dietary administration of oxytetracycline (OTC) on the dynamic of the gut microbial community and the integrity of the intestinal structure were evaluated in Nile tilapia (*Oreochromis niloticus*), and different diets were used to access the recovery after OTC treatment. To evaluate the direct effects of the medicated feed, two experimental groups were established: the control group (C), fed with a commercial-type diet; and the OTC group (O), fed with a commercial-type diet supplemented with OTC at 55 mg Kg^{-1} body weight day^{-1}. The trial lasted for 10 days. After medication, the recovery was evaluated for 30 days. The control group remained fed with the commercial-type diet (C group), while the O group was divided into two groups. Half was fed with the control diet (OC group), and the other half was fed with a high plant-based diet (OP group). Several samplings were performed, primarily to evaluate the direct effects of OTC (day 10) and then, to evaluate recovery (days 18, 25, and 40). Culturable bacteria, microbiota community, and intestinal histology were assessed. *Vibrio* counts decreased significantly in the posterior intestine of the O group after feeding with OTC. OTC did not produce significant variations in the intestinal microbiota community indices; however, there was a shift to *Proteobacteria*, *Fusobacteria*, and *Firmicutes* phyla in the anterior intestine, and to *Bacteroidetes*, *Verrucomicrobia*, and *Fusobacteria* phyla in the posterior intestine, with OTC treatment. The antibiotic-induced modulation of the microbiota was very distinct between the intestinal portions, with *Legionella, Bacillus, Leucobacter, Aeromicrobium* and *Kocuria* genera, affected in the anterior intestine, and *Bacteroides* genus in the posterior intestine. The recovery diets helped to restore up to 56% (control diet) or 60% (high plant-based diet) of the antibiotic-induced modifications on the microbiota dynamics, and no pathogenic-associated bacteria found exclusively in the O group emerged as unique with the recovery diets. The dietary administration of OTC did not produce significant changes in the intestinal structure, and, though high plant protein-based diets can induce intestinal inflammation, no major changes were verified in OP group at the end of the trial. Nile tilapia presents a huge intestinal adaptability and was able to recover from an antibiotic treatment even with a challenging high plant-based diet.

1. Introduction

The gastrointestinal tract of fish is a complex multifunctional organ that plays an important role in fish health and survival, being responsible for physiologic and metabolic processes and helping the immune system against pathogens (Salinas & Parra, 2015; Limbu et al., 2018; Almeida et al., 2019). In fish farming, diets can be used to enhance fish immunity and health, through the manipulation of the intestinal structure and microbial community (improving survival and growth), and as a vehicle of antimicrobials for the control of bacterial diseases (Navarrete et al., 2008; Salinas & Parra, 2015). Nile tilapia (*Oreochromis niloticus*) is one of the most produced freshwater fish worldwide (FAO, 2020), due to its fast growth rates and resistance to environmental conditions (FAO, 2005-2021). Still, tilapia production suffers mortalities and economic losses associated with bacterial diseases (Rigos & Troisi, 2005; Sekkin & Kum, 2011). Antibiotics are very useful to control bacterial infections, however, they can select resistant bacteria, induce intestinal toxicity, and stimulate dysbiosis (imbalance between commensal and pathogenic bacteria) (Navarrete et al., 2008, El-Adawy et al., 2018; Limbu et al., 2018; Almeida et al., 2019; Julinta et al., 2019). One of the most used antibiotics in aquaculture is oxytetracycline (OTC), due to its broad-spectrum bacteriostatic activity, and is normally incorporated in the feed at a dose of 75 mg kg^{-1} body weight day^{-1} (varying between 10 - 100 mg) for 7-10 days (Rigos & Troisi, 2005; Sekkin & Kum, 2011). In Portugal, the recommended dose is 55 mg kg^{-1} body weight day^{-1}, for 7-10 days, for the treatment of *Lactococcus garvieae*, *Aeromonas hydrophila* and *Vibrio anguillarum* diagnosed infections (DGAV, 2020).

Aquafeed formulation relies on fish meal and fish oil as protein and lipid sources, however, the pressure on the available supplies and the escalating prices, are demanding sustainable alternatives (Al-Kenawy et al., 2008; Kuli Khan et al., 2013). Vegetable ingredients are sustainable and cheaper alternatives, but they have been associated with an imbalanced amino acid profile, high amounts of fibre and non-starch polysaccharides, and anti-nutritional factors (Hassaan et al, 2018). Anti-nutritional factors are known to induce intestinal damage and inflammation, contributing to poor absorption, increased mucus production, and enhanced leukocyte infiltration, especially in carnivorous fish (Estruch et al., 2018; Kumar et al., 2020). In Nile tilapia aquaculture, plant protein sources, such as soybean meal, are being used successfully in feed formulations (Al-Kenawy et al., 2008; Kuli Khan et al., 2013; Ogello et al., 2014; Hassaan et al, 2018).

Considering that a single course of antibiotics can change the microbial community of the gut, and diets can be used to manipulate the intestinal structure and microbiota, this study aimed to evaluate the effects of the dietary administration of OTC on the integrity of the intestinal structure and its influence on the dynamic of the microbial community, as well as, to

understand the influence of different diets (commercial-type and high plant-based diet) in the intestinal recovery after challenging with an antibiotic.

2. Material and methods

2.1. Fish and acclimatization period

Following FELASA category B and C recommendations and according to the European and Portuguese guidelines on the protection of animals used for scientific purposes “Directive 2010/63/UE” and “Decreto-Lei n.º 113/2013 de 7 de Agosto”, the trial was performed at UTAD (University of Trás-os-Montes e Alto Douro) facilities, Vila Real, Portugal. From Til-Aqua International (The Netherlands), 315 Nile tilapias, weighing 118 ± 0.8 g were randomly distributed among 9 tanks with 300 L of water capacity (35 fish each tank), in a freshwater recirculating system. A photoperiod of 10 h light 14 h $dark^{-1}$ was applied. Water dissolved oxygen was maintained above 90% and the water temperature was 23 ± 0.5 ºC. All animals were acclimatized to the experimental conditions, for 15 days and fed with a control diet (a commercial-type diet that fulfilled the known nutritional requirements for Nile tilapia) (Table 1). The diet was produced by Sparos Lda. (Olhão, Portugal) and the fish were fed twice a day, to apparent visual satiety.

Table 1: Ingredient and chemical composition of the diets.

Ingredients (% feed)*	Control diet	Antibiotic diet	Plant diet
Fishmeal 60	5.0	5.0	
Poultry meal 65	5.0	5.0	
Wheat gluten meal	0.5	0.5	0.5
Corn gluten meal	7.0	7.0	7.0
Soybean meal 44	22.0	22.0	22.0
Soybean meal full fat			11.7
Sunflower meal			11.7
Wheat meal	15.0	15.0	12.3
Wheat bran	9.0	9.0	9.0
Rice bran full fat	12.0	12.0	12.0
Corn meal	15.6	15.6	5.7
Soybean oil	4.6	4.6	4.0
Vitamin and mineral premix	1.0	1.0	1.0
Antioxidant	0.2	0.2	0.2
Sodium propionate	0.1	0.1	0.1
Monoammonium phosphate	1.0	1.0	1.0
L-Histidine	0.2	0.2	0.2
L-Lysine	0.8	0.8	0.8
L-Threonine	0.2	0.2	0.2
L-Tryptophan	0.2	0.2	0.2
L-Valine	0.2	0.2	0.2
DL-Methionine	0.4	0.4	0.4
Oxytetracycline		0.3	
Proximate analyses (% dry matter)			
Crude protein	29.0		29.0
Crude fat	10.0		10.0
Fibre	3.9		6.8
Starch	25.4		17.6
Ash	6.5		6.2
Gross energy	18.9		19.0

***Fishmeal 60:** CONRESA 60 - 65 % CP, 10 % CF, Conserveros Reunidos S.A., Spain; **Poultry meal 65:** 65 % CP, 12 % CF, SAVINOR UTS, Portugal; **Wheat gluten meal:** VITEN - 81 % CP, 2.1 % CF, Roquette, France; **Corn gluten meal:** 58 % CP, 4 % CF, MPS, France; **Soybean meal 44:** solvent extracted soybean meal - 43 % CP, 2.7 % CF, CARGILL, Spain; **Soybean meal full fat:** 36 % CP, 17 % CF, Ribeiro & Sousa Lda, Portugal; **Sunflower meal:** solvent extracted dehulled sunflower meal - 43 % CP, 3 % CF, MAZZOLENI SPA, Italy; **Wheat meal:** 10.2 % CP; 1.2 % CF, MOLISUR, Spain; **Wheat bran:** 14.8 % CP, 4.7 % CF, Ribeiro e Sousa Lda, Portugal; **Rice bran full fat:** 13.3 % CP; 16.3 % CF, Ribeiro e Sousa Lda, Portugal; **Corn meal:** 8.6 % CP; 4.3 % CF, Casa Lanchinha, Portugal; **Soybean oil:** J.C. Coimbra, Portugal; **Vitamin and mineral premix:** PREMIX Lda, Portugal - Vitamins (IU or mg kg^{-1} diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 500 mg; inositol, 500 mg; biotin, 3 mg; calcium pantothenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg kg^{-1} diet): copper sulfate, 9 mg; ferric sulfate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulfate,7.5 mg; sodium chloride, 400 mg; excipient wheat middling's; **Antioxidant:** VERDILOX PX, Kemin Europe NV, Belgium; **Sodium propionate:** Premix Lda, Portugal; **Monoammonium phosphate:** Windmill Aquaphos - 26 % P, Aliphos Rotterdam B.V, The Netherlands; **L-Histidine:** 99 %, Sigma-Aldrich, USA; **L-Lysine:** 99 %: Ajinomoto Eurolysine SAS, France; **L-Threonine:** 98.5 %, Evonik Nutrition & Care GmbH, Germany, **L-Tryptophan:** 98 %, Evonik Nutrition & Care GmbH, Germany; **L-Valine:** 98 %, Evonik Nutrition & Care GmbH, Germany; **DL-Methionine:** Rhodimet NP99, ADISSEO, France; **Oxytetracycline:** Aquacen Cloridrato Oxitetraciclina 1000 mg g^{-1}, Cenavisa, Spain.

2.2. Experimental design

From the 9 tanks used in the acclimatization period, animals from 3 tanks remained fed with the commercial-type diet (C group), whereas the animals from the other 6 tanks shift to the medicated feed with OTC (O group) at a dose of 55 mg Kg^{-1} body weight day^{-1} (DGAV, 2020) (Figure 1). The medicated feed contained 0.275% of OTC, applied by vacuum coating in the control diet. This trial lasted for 10 days. Afterwards, the recovery (with different diets) was evaluated: the C group (3 tanks) remained fed with the control diet, and the O group was divided: half (3 tanks) were fed the control diet (OC group), and the other half (3 tanks) were fed a high plant-based diet (OP group) (Figure 1). This trial lasted for 30 days. Nile tilapia juveniles were fed twice a day, until visual apparent satiety.

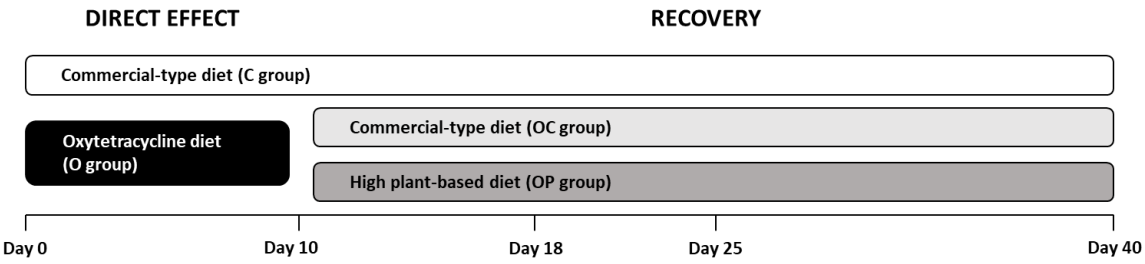

Figure 1: Experimental design. Evaluation of the effects of oxytetracycline medicated feed at day 10 (direct effect) and at days 18, 25, and 40 (recovery).

2.3. Intestine collection

The structural intestinal effects of the OTC were evaluated on day 10 (direct effect) and on days 18, 25, and 40 of the recovery period. Nine fish per tank were randomly selected, euthanized by immersion in 2-phenoxyethanol (1500 ppm; Sigma), and sampled for intestinal tissue in sterile conditions. The fish were dissected, and the internal organs were placed in a petri dish. Sections of 1 cm of the anterior and posterior intestine and the respectively digesta were collected for classical culture microbiology and stored at 2-8 ºC until assayed. Two cm portions of the anterior and posterior intestine and the respectively digesta were collected and stored at - 20 ºC in 96% molecular-grade ethanol for microbiota analysis until assayed. Sections of 2 cm of the anterior and posterior intestine were also collected, flushed with sodium phosphate buffer, and fixated in a 4% buffered formalin solution (Sigma) until assayed.

2.4. Culture-dependent microbiology

Culturable bacteria were identified by classical culture techniques, following ISO 6887-2:2003, NP 3005:1985, and ISO 4833:2003 standards, and Naghili et al, 2013 recommendations, with some modifications. Briefly, 100 mg of intestinal samples (n = 3) from the same tank were pooled and homogenized with 900 µL of Buffered Peptone Water (BIOKAR Diagnostics, France) (10^{-1} dilution). Afterward, 100 µL of this suspension were serially diluted in 900 µL of Tryptone Salt Broth (BIOKAR Diagnostics, France) (until 10^{-8} dilution). Ten µL of each dilution was then cultured on Tryptic Soy Agar 1.5% NaCl (TSA, isolation of total culturable bacteria, Condalab, Spain), Thiosulfate Citrate Bile Salts Sucrose Agar (TCBS, isolation of *Vibrio* spp., Condalab, Spain) and Aeromonas agar (isolation of *Aeromonas* spp., Condalab, Spain), in triplicates, employing the microdrop technique. The plates were incubated for 24 or 48 hours, and the colony-forming units (CFUs) were measured in the highest dilution with less than 30 colonies.

2.5. Microbiota analysis by NGS

DNA extraction from intestinal samples was performed as described by Serra et al. (2021), with some modifications. Briefly, the intestinal samples from the same tank were pooled (n = 3), suspended, and homogenized as described by Serra et al. (2021), however, the homogenization was performed 3 times. Following the incubations performed according to Serra et al. (2021), and centrifugations at 13000 rpm for 10 min, only 10 mg ml^{-1} of lysozyme (Sigma) were used. Later, the DNA pellet was washed twice with 500 µl of ice-cold 70% ethanol by centrifugation (13000 rpm, 10 min) and dried at 37 ºC for 10 min. The DNA was resuspended in 50 µl ultrapure water for 30 min at 37 ºC and stored at -20 °C. Following the manufacturer recommendations, an additional purification step was performed using the OneStep™ PCR Inhibitor Removal Kit (Zymo Research).

The taxonomic diversity of the intestinal microbiota was assessed by next-generation sequencing (NGS) technology, using the sequences obtained by Illumina MiSeq platform (Macrogen Inc., Seoul, Rep. of Korea), targeting the V3-V4 hypervariable region of the 16S rRNA gene, to obtain a sequence informative length of 300 bp. The paired-end reads were merged to produce longer reads using the Flash program (Magoc & Salzberg, 2011). The pre-processing (e.g. removal of low-quality reads) and clustering (Li & Godzik, 2006) were performed using the CD-HIT program. The filtered sequences were clustered at 100% identity into operational taxonomic units (OTU) identifying the chimeric reads, and after the removal of the noise sequences (small size), the remaining representative reads from non-chimeric clusters were clustered into OTUs at a 97% ID to species level cut-off. Singletons and low

abundant (< 8) OTUs were removed from the downstream analysis. Taxonomy assignment and diversity statistics were accessed by Quantitative Insights Into Microbial Ecology (QIIME) software (Caporaso et al., 2010) and the SILVA138 16S reference database (Quast et al., 2013). STAMP v2.1.3 software (Beikolab) (Parks et al., 2014) was used to evaluate the metagenomic profiles while Venn diagrams were obtained employing Venny 2.1 software (BioinfoGP) (Oliveros, 2007-2015).

2.6. Histology

Following Guardiola et al. (2013) recommendations, the intestinal portions were fixated, dehydrated, and placed in a xylene solution for transparency. Afterward, the samples were embedded in paraffin and sectioned transversally into 2 µm sections. The intestinal sections were mounted and stained with hematoxylin-eosin (H-E) solutions, and villi height, width, and area, and the number of caliciform cells were measured using a light microscope (Nikon Eclipse E200) equipped with a camera (Sony, E3CMOS06300KPA) and appropriate software (ToupView 3.7 for a digital camera).

2.7. Statistical analysis

Results were expressed as means (M) and standard deviation (SD). Data were analysed for normality and homogeneity of variance and, when necessary, log-transformed before being treated statistically, using one-way ANOVA followed by the Tukey post hoc test to identify differences in the experimental treatments or intestinal portions, within each time point tested. Statistical analysis was performed using IBM SPSS Statistics 24 and STAMP v2.1.3 software (Beikolab) (Parks et al., 2014). The level of significance used was $p < 0.05$ for all statistical tests.

3. Results

3.1. Culturable microbiota analysis

Total culturable bacteria and *Aeromonas* counts were not significantly affected by the OTC. On contrary, *Vibrio* counts in the posterior intestine decreased significantly in the O group (0.0 ± 0.0 CFUs ml^{-1}) compared to the C group (338.9 ± 18.4 CFUs ml^{-1}). The recovery diets (OC and OP groups) did not influence the number of culturable bacteria during the recovery period. Comparing both portions of the intestine, the posterior portion usually presented a higher degree of culturable bacteria than the anterior segment.

3.2. Total microbiota analysis

Nile tilapia intestinal microbiota structure was assessed by 16S rRNA amplicon sequencing using the Illumina Miseq sequencing platform. At least 40235 read counts were obtained per sample (Supplementary Table S1), and after pre-processing, a total of 2087189 high-quality reads were clustered into 976 OTUs at a 97% identity threshold (Supplementary Table S1). The total of sequences obtained from both portions of the intestine was assigned to 17 phyla, 46 classes, 107 orders, 229 families, and 559 genera. Unclassified bacteria represented up to 4% of the total sequencing reads. Classified taxa with a proportion of 1% or higher were considered the most abundant (Figure 2).

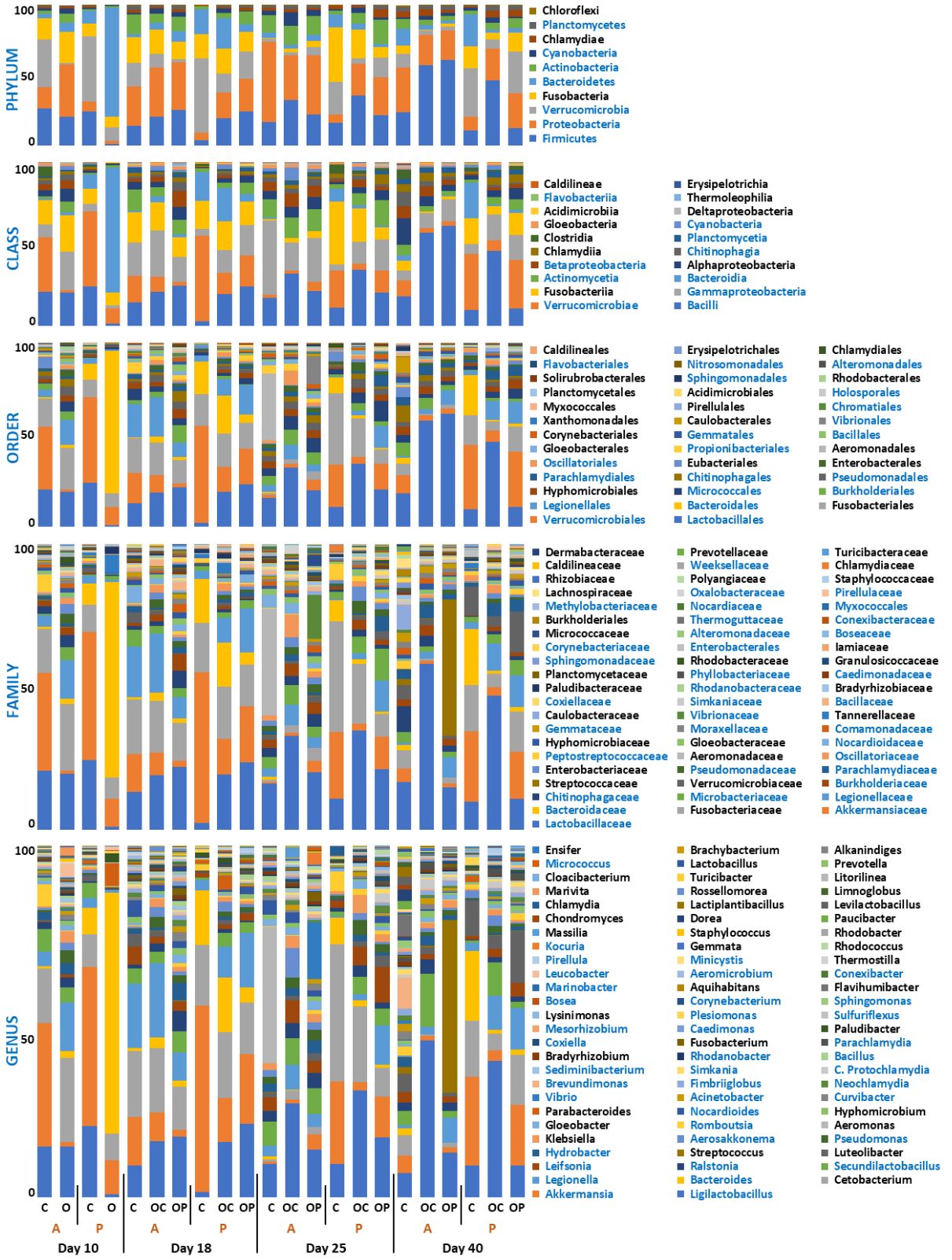

Figure 2: Relative bacterial abundance (y-axis) found in the anterior (**A**) and posterior (**P**) intestine of Nile tilapia fed with oxytetracycline medicated feed for 10 days. Evaluation of the direct effects (day 10) and recovery (days 18, 25, and 40). **C** (control diet), **O** (oxytetracycline diet), **OC** (oxytetracycline + control diet), **OP** (oxytetracycline + plant diet). Presented are taxa with a proportion ≥1% in any experimental feeding condition. Taxa in **blue** were significantly different among the dietary treatments ($p < 0.05$) (sampling point not specified).

The OTC (day 10) did not produce significant variations in the overall intestinal microbiota community indices; however, the anterior segment demonstrated a higher diversity and evenness than the posterior segment in the O group (highlighted by orange letters) (Figure 3).

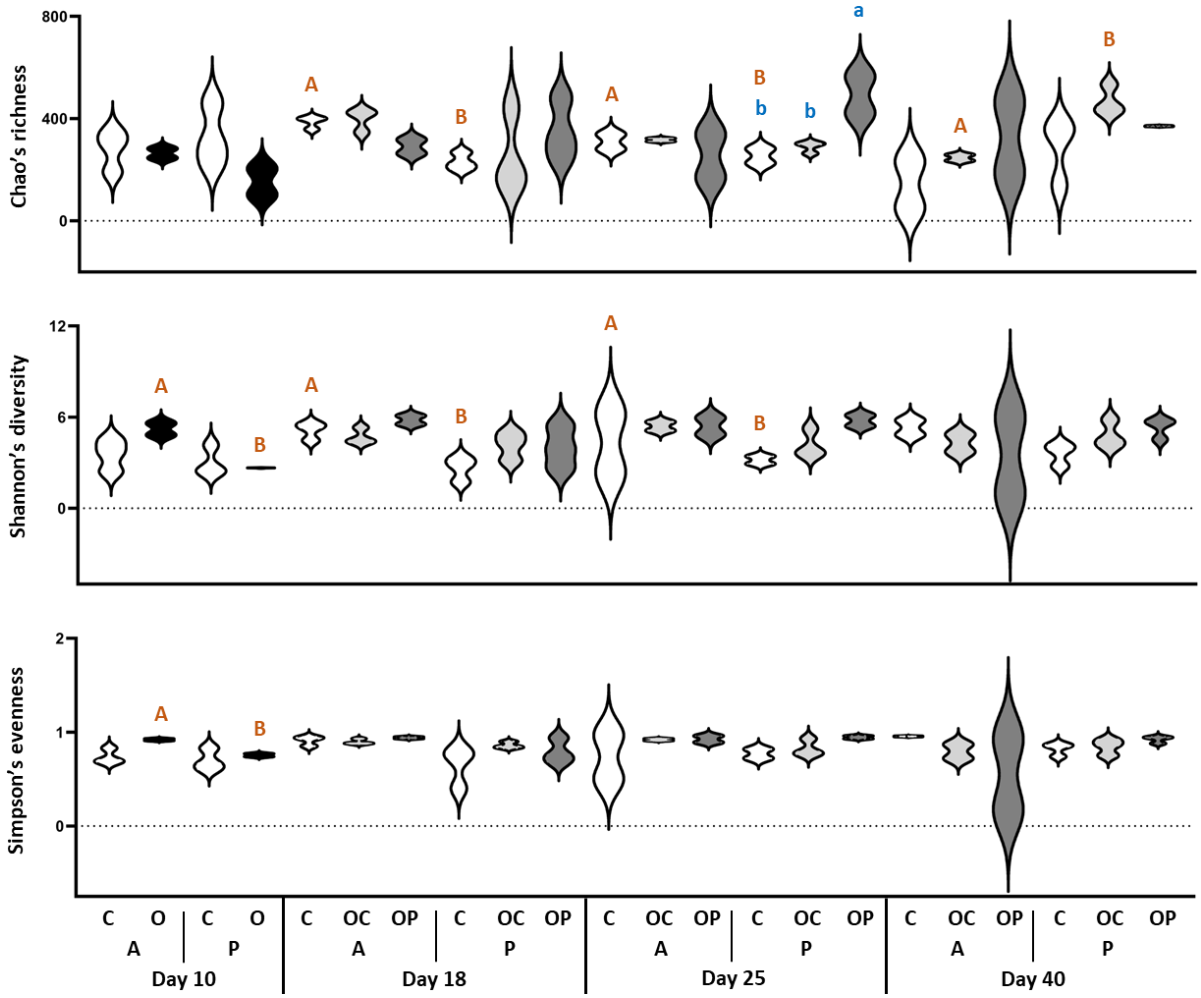

Figure 3: Gut microbiota community richness (Chao), diversity (Shannon) and evenness (Simpson) indices, obtained from NGS analysis of the Nile tilapia anterior (**A**) and posterior (**P**) intestinal microbiota after a medicated feed with oxytetracycline has been given for 10 days. Evaluation of the direct effects (day 10) and recovery (days 18, 25, and 40). **C** (control diet), **O** (oxytetracycline diet), **OC** (oxytetracycline + control diet), **OP** (oxytetracycline + plant diet). Different **blue letters** mean significant differences among the dietary treatments in that intestinal portion ($p < 0.05$). The **orange letters** represent significant differences between the intestinal portions in that diet ($p < 0.05$).

Nevertheless, OTC induced variations in the microbiota community composition of both portions of the intestine (Figure 2). For example, Phyla associated with the C group in the anterior intestine (*Verrucomicrobia*, *Firmicutes*, and *Proteobacteria*) and in the posterior intestine (*Verrucomicrobia*, *Firmicutes*, and *Fusobacteria*), upon the antibiotic treatment shifted to *Proteobacteria*, *Fusobacteria*, and *Firmicutes* in the anterior intestine, and to *Bacteroidetes*, *Verrucomicrobia*, and *Fusobacteria* in the posterior intestine (Figure 2).

Comparing both portions of the intestine, the microbiota response to the antibiotic was very distinct, with the establishment of different bacterial communities in the two portions of the intestine. In the anterior intestine, the antibiotic-induced modulation of the microbiota was very variable, while in the posterior intestine, most of the taxa were reduced (87%) (Figure 2 and 4, Supplementary Table S2).

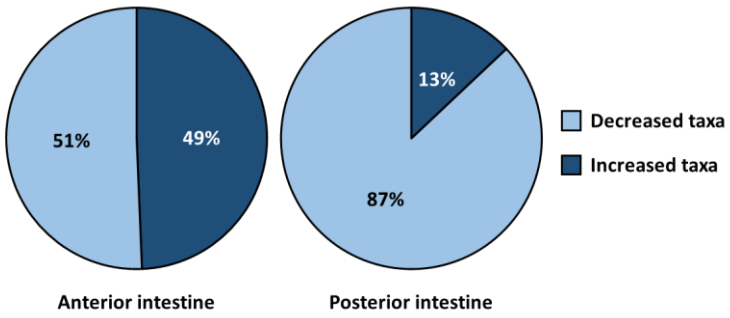

Figure 4: Oxytetracycline direct effects on taxa abundance in Nile tilapia's intestinal microbiota after the dietary administration of medicated feed for 10 days, with distinct response in the intestinal portions. Variable response in the anterior intestine and reduction of taxa in the posterior intestine.

At genus level, in the anterior intestinal segment, OTC significantly affected *Legionella*, *Bacillus*, *Leucobacter*, *Aeromicrobium* and *Kocuria* genera, while in the posterior intestine, it only affected the *Bacteroides* genus (Table 2 and 3, Figure 2, Supplementary Table S2).

Table 2: Taxa abundance (%) at Genus Taxonomic level, obtained from NGS analysis of the Nile tilapia **anterior** intestinal microbiota after feeding the medicated feed with oxytetracycline for 10 days. Evaluation of the direct effects (day 10) and recovery (days 18, 25, and 40). **C** (control diet), **O** (oxytetracycline diet), **OC** (oxytetracycline + control diet), **OP** (oxytetracycline + plant diet). Values are presented as means ± standard deviation. Presented are taxa with a mean proportion ≥1% in the experimental feeding conditions. Different **letters** mean significant differences among the dietary treatments in that intestinal portion ($p < 0.05$).

Day	Phylum	Class	Order	Family	Genus	Diet		
						C	O	
10	Proteobacteria	Gammaproteobacteria	Legionellales	Legionellaceae	Legionella	3.8 ± 1.3[b]	12.3 ± 1.0[a]	
	Actinobacteria	Actinomycetia	Micrococcales	Microbacteriaceae	Leucobacter	0.0 ± 0.0[b]	3.3 ± 1.3[a]	
						C	OC	OP
18	Actinobacteria	Actinomycetia	Micrococcales	Microbacteriaceae	Leifsonia	4.6 ± 0.5[a]	1.9 ± 0.5[b]	2.2 ± 0.4[b]
	Bacteroidetes	Chitinophagia	Chitinophagales	Chitinophagaceae	Hydrobacter	1.0 ± 0.5[b]	1.0 ± 0.5[b]	4.4 ± 0.9[a]
	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Ralstonia	1.0 ± 0.5[b]	2.2 ± 1.0[b]	5.1 ± 1.0[a]
	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Curvibacter	0.5 ± 0.2[b]	0.5 ± 0.3[b]	2.2 ± 0.3[a]
	Actinobacteria	Actinomycetia	Corynebacteriales	Corynebacteriaceae	Corynebacterium	0.1 ± 0.0[b]	0.1 ± 0.1[b]	1.2 ± 0.5[a]
25	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Ligilactobacillus	8.7 ± 0.3[b]	23.9 ± 0.8[a]	12.0 ± 0.6[b]
40	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Secundilactobacillus	3.6 ± 0.9[b]	13.8 ± 1.0[a]	1.9 ± 1.9[b]
	Firmicutes	Clostridia	Eubacteriales	Peptostreptococcaceae	Romboutsia	2.2 ± 0.2[a]	0.3 ± 0.0[b]	0.2 ± 0.0[b]

Table 3: Taxa abundance (%) at Genus Taxonomic level, obtained from NGS analysis of the Nile tilapia **posterior** intestinal microbiota after feeding the medicated feed with oxytetracycline for 10 days. Evaluation of the direct effects (day 10) and recovery (days 18, 25, and 40). **C** (control diet), **O** (oxytetracycline diet), **OC** (oxytetracycline + control diet), **OP** (oxytetracycline + plant diet). Values are presented as means ± standard deviation. Presented are taxa with a mean proportion ≥1% in the experimental feeding conditions. Different **letters** mean significant differences among the dietary treatments in that intestinal portion ($p < 0.05$).

Day	Phylum	Class	Order	Family	Genus	Diet		
						C	O	
10	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	7.3 ± 4.7[b]	68.1 ± 1.1[a]	
						C	OC	OP
18	Actinobacteria	Thermoleophilia	Solirubrobacterales	Conexibacteraceae	Conexibacter	0.0 ± 0.0[b]	0.0 ± 0.0[b]	0.6 ± 0.3[a]
25	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	7.4 ± 1.5[a]	1.7 ± 0.8[b]	0.7 ± 0.6[b]
	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Akkermansiaceae	Akkermansia	23.0 ± 4.2[a]	2.3 ± 0.4[b]	10.3 ± 2.7[b]
	Proteobacteria	Gammaproteobacteria	Legionellales	Legionellaceae	Legionella	0.5 ± 0.0[b]	1.7 ± 1.3[b]	9.7 ± 3.6[a]
	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	0.2 ± 0.1[b]	1.0 ± 0.2[a]	0.6 ± 0.1[ab]
	Proteobacteria	Gammaproteobacteria	Legionellales	Coxiellaceae	Coxiella	0.1 ± 0.0[b]	0.4 ± 0.3[b]	1.0 ± 0.0[a]
	Cyanobacteria	Cyanobacteria	Oscillatoriales	Oscillatoriaceae	Aerosakkonema	0.3 ± 0.2[b]	1.9 ± 0.5[a]	0.5 ± 0.1[b]
	Chlamydiae	Chlamydiia	Parachlamydiales	Parachlamydiaceae	Candidatus Protochlamydia	0.1 ± 0.0[b]	0.3 ± 0.3[b]	2.2 ± 0.1[a]
	Chlamydiae	Chlamydiia	Parachlamydiales	Parachlamydiaceae	Neochlamydia	0.0 ± 0.0[b]	0.3 ± 0.1[b]	1.8 ±0.6[a]
	Actinobacteria	Actinomycetia	Propionibacteriales	Nocardioidaceae	Nocardioides	0.1 ± 0.0[b]	0.2 ± 0.1[b]	1.8 ± 0.3[a]
40	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Akkermansiaceae	Akkermansia	24.3 ± 9.4[a]	2.7 ± 1.1[b]	15.6 ± 3.6[ab]
	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Ligilactobacillus	8.8 ± 10.3[b]	35.7 ± 9.7[a]	8.3 ± 3.6[b]
	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Secundilactobacillus	0.5 ± 0.5[b]	8.6 ± 1.2[a]	1.7 ± 0.1[b]
	Proteobacteria	Gammaproteobacteria	Enterobacterales	Enterobacterales	Plesiomonas	2.1 ± 0.9[a]	0.3 ± 0.2[b]	0.4 ± 0.5[b]
	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	0.4 ± 0.3[b]	1.7 ± 0.6[a]	0.8 ± 0.2[b]
	Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	Marinobacter	0.2 ± 0.0[b]	0.5 ± 0.1[ab]	0.8 ± 0.3[a]
	Actinobacteria	Actinomycetia	Micrococcales	Microbacteriaceae	Leifsonia	0.9 ± 0.7[b]	2.2 ± 0.4[ab]	3.6 ± 0.9[a]
	Chlamydiae	Chlamydiia	Parachlamydiales	Simkaniaceae	Simkania	0.2 ± 0.1[b]	0.7 ± 0.4[ab]	1.1 ± 0.3[a]

Also, less unique genera (n = 9; 4.3%) were found in the posterior intestine of the O group compared to the C group (n = 62; 51.7%) (Table 4). *Levilactobacillus*, *Latilactobacillus*, *Clostridium*, *Bifidobacterium*, *Roseomonas*, *Rhodococcus*, *Rothia*, *Kocuria*, *Prevotella*, *Parabacteroides*, *Shewanella*, *Agromyces*, *Empedobacter*, *Aurantimonas*, *Selenomonas*, *Oligella*, *Delftia*, *Treponema*, and *Halomonas* were some of the genera found exclusively in the O group.

Table 4: Shared and unique genera found in anterior (**A**) and posterior (**P**) intestinal samples of the Nile tilapia after feeding the medicated feed with oxytetracycline for 10 days. Evaluation of the direct effects (day 10) and recovery (days 18, 25, and 40). **C** (control diet), **O** (oxytetracycline diet), **OC** (oxytetracycline + control diet), **OP** (oxytetracycline + plant diet).

Shared and unique genera						
Direct effect	**Day 10**					
	A	**P**				
Common in C and O	139 (51.5 %)	92 (44.0 %)				
Exclusively in C	69 (25.6 %)	108 (51.7 %)				
Exclusively in O	62 (23.0 %)	9 (4.3 %)				
Recovery	**Day 18**		**Day 25**		**Day 40**	
	A	**P**	**A**	**P**	**A**	**P**
Common in C, OC and OP	145 (45.2%)	120 (46.2 %)	130 (47.6 %)	122 (43.9 %)	99 (37.4 %)	149 (51.7 %)
Common in C and OC	33 (10.3 %)	4 (1.5 %)	19 (7.0 %)	13 (4.7 %)	7 (2.6 %)	13 (4.5 %)
Common in C and OP	18 (5.6 %)	11 (4.2 %)	13 (4.8 %)	11 (4.0 %)	15 (5.7 %)	14 (4.9 %)
Common in OC and OP	22 (6.9 %)	36 (13.8 %)	19 (7.0 %)	44 (15.8 %)	36 (13.6 %)	36 (12.5 %)
Exclusively in C	32 (10.0 %)	10 (3.8 %)	28 (10.3 %)	10 (3.6 %)	18 (6.8 %)	8 (2.8 %)
Exclusively in OC	37 (11.5 %)	35 (13.5 %)	30 (11.0 %)	32 (11.5 %)	26 (9.8 %)	36 (12.5 %)
Exclusively in OP	34 (10.6 %)	44 (16.9 %)	34 (12.5 %)	46 (16.5 %)	64 (24.2 %)	32 (11.1 %)

In the recovery phase of the trial, only the intestinal microbiota richness index differed between the dietary treatments, at day 25, in the posterior intestine (represented by blue letters), and between the intestinal portions (highlighted by orange letters) (Figure 3). Still, the microbial community presented numerous adjustments during the recovery trial, with several taxa being significantly affected by the dietary treatments (Figure 2).

Comparing the dietary effects of the OTC (day 10) in both portions of the intestine, and the recovery effects of the experimental diets at the end of the trial (day 40), we could verify that the microbial community present in fish fed the antibiotic (represented by squares in Figure 5) differ from that present in fish fed the recovery dietary treatments (represented by diamonds and hexagons in Figure 5), especially in the posterior intestine.

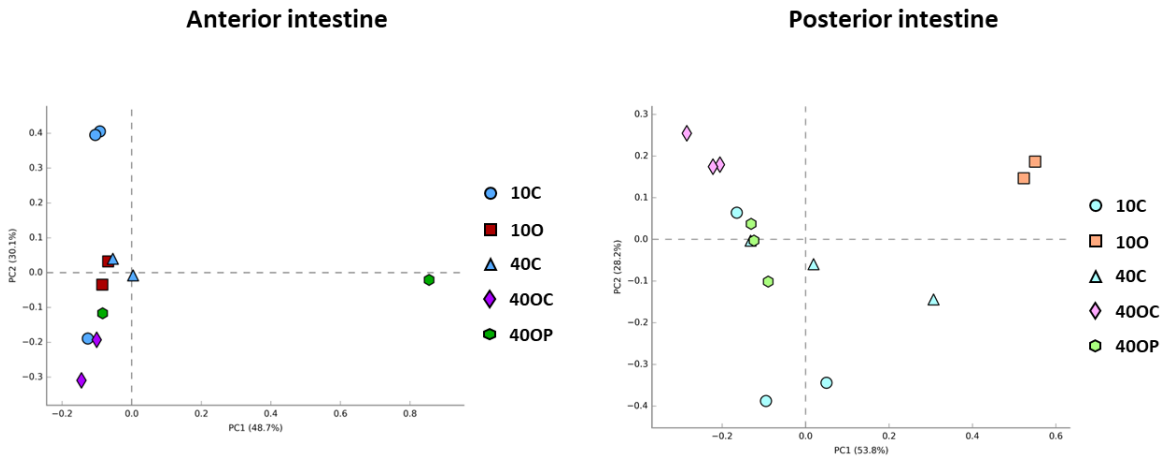

Figure 5: Principal component analysis (PCA) of the oxytetracycline effects at day **10** (direct effect) and at the end of the trial - day **40** (recovery) in the intestinal microbiota (at Genus Taxonomic level) of Nile tilapia. **10C** (day 10, control diet), **10O** (day 10, oxytetracycline diet), **40C** (day 40, control diet), **40OC** (day 40, oxytetracycline + control diet), **40OP** (day 40, oxytetracycline + plant diet).

In the anterior intestine, only 45% of the variations induced in the genus taxa by the antibiotic were neutralized with the control diet (OC group), while 55% of the affected taxa recovered with the plant diet (OP group). Likewise, in the posterior intestine, 56% of the affected taxa recovered with the control diet (OC group), whereas 60% of the variations were ameliorated with the plant diet (OP group) (Figure 6, Supplementary Table S2).

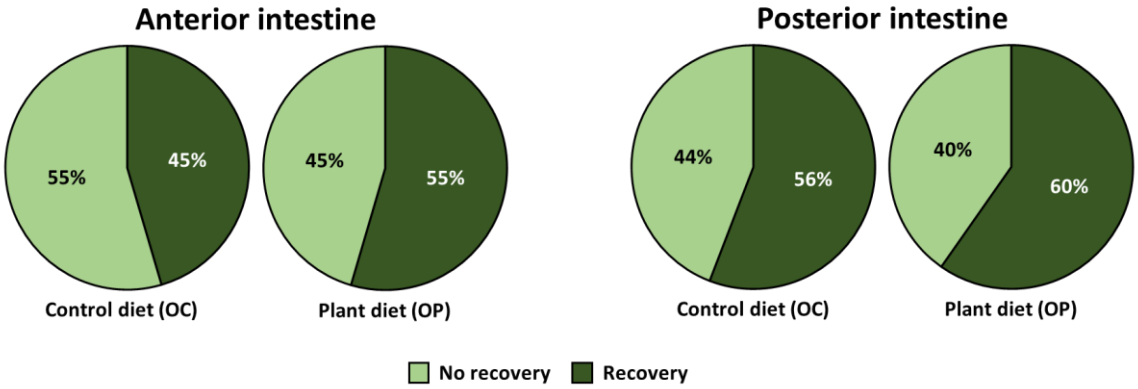

Figure 6: Dietary modulation of Nile tilapia's intestinal microbiota in the recovery period: % of taxa exhibiting recovery (increase in abundance) from the oxytetracycline treatment, upon feeding a control diet (OC group) or a plant diet (OP group), in both portions of intestine.

Leifsonia, *Hydrobacter*, *Ralstonia*, *Curvibacter*, *Corynebacterium, Ligilactobacillus*, *Secundilactobacillus* and *Romboutisia*, were the main affected genera by the recovery dietary treatments in the anterior intestine, while *Conexibacter*, *Bacteroides*, *Akkermansia*, *Legionella*, *Acinetobacter*, *Coxiella*, *Aerosakkonema*, *Candidatus Protochlamydia*, *Neochlamydia*, *Nocardioides*, *Ligilactobacillus*, *Secundilactobacillus*, *Plesiomonas*, *Pseudomonas*, *Marinobacter*, *Leifsonia and Simkania* were the main affected genera in the posterior intestine (Tables 2 and 3).

In the recovery trial, the unique genera found in the anterior intestine for each dietary treatment, were similar in each sampling point, except in day 40, where 24.2% of the exclusive genera were found in the OP group, against the 6.8% found in the C group. On the other hand, in the posterior intestine, the OP group presented more exclusive genera than the C group (Table 4).

From all the genera found in the recovery diets, in all the sampling points, most of the genera were shared by the dietary treatments (56.3%). However, the OC group presented 9.2% of unique genera and 15.4% of exclusive genera were also found in the OP group (Figure 7). *Abiotrophia*, *Brevibacillus*, *Chryseobacterium*, *Finegoldia*, *Herbaspirillum*, *Priestia*, *Rhizobacter*, *Stenotrophomonas*, *Thermoanaerobacterium*, were some of the genera found exclusively in the OC group; and *Aerococcus*, *Arcobacter*, *Arthrobacter*, *Brucella*, *Chitinophaga*, *Dialister*, *Flavonifractor*, and *Mycolicibacter* were the unique genera found in the OP group.

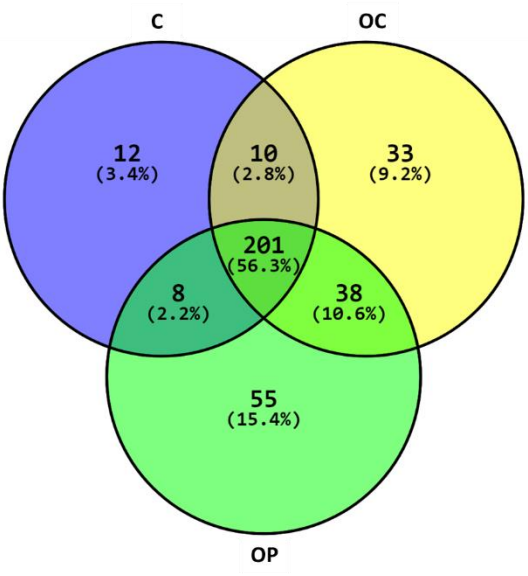

Figure 7: Venn´s diagram of shared and unique genera found in the experimental diets in the recovery period (all sampling points). **C** (control diet), **OC** (oxytetracycline + control diet), **OP** (oxytetracycline + plant diet).

3.3. Histology

Nile tilapia´s intestines were very long and coiled, with apparently different morphology in the anterior and posterior sections, but with no macroscopic variations between the dietary treatments (Figure 8).

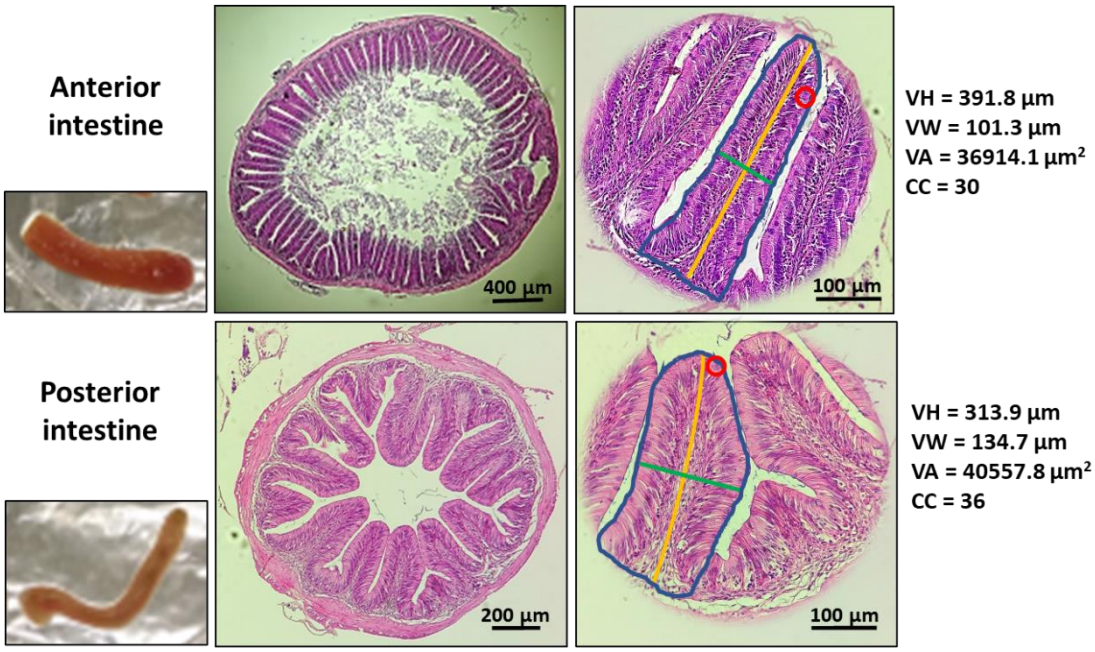

Figure 8: Anterior and posterior intestinal examples of Nile tilapia´s intestines. Histomorphology elements evaluated, villi height (yellow line), villi width (**green line**), villi area (**blue line**), and caliciform cells (**red circle**).

Microscopically, OTC (day 10) did not induce significant alterations in the integrity of the intestine wall (absence of blue letters), but the anterior portion of the intestine presented higher villi height and area than the posterior, in both dietary groups (highlighted by orange letters) (Figure 9).

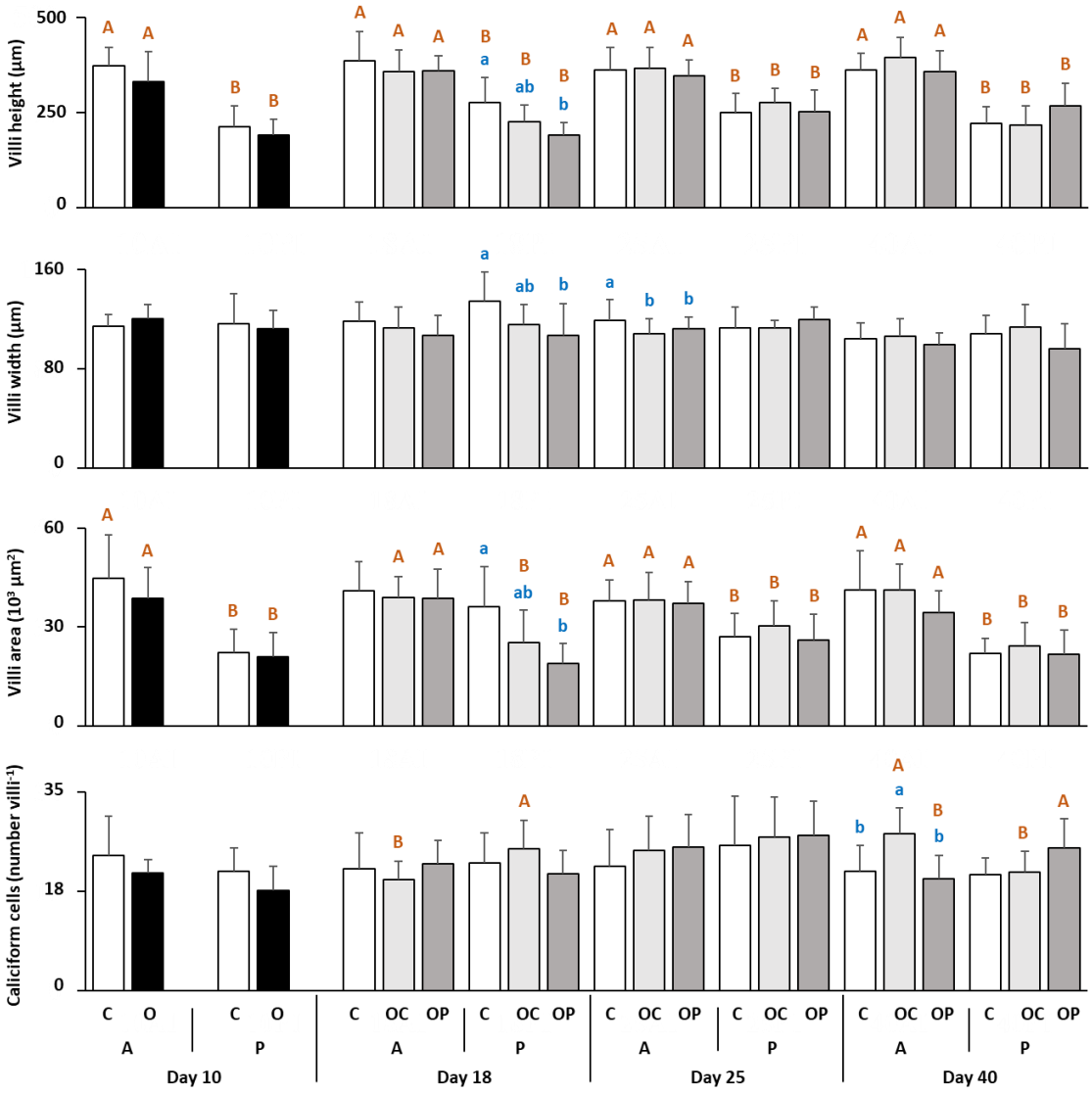

Figure 9: Histological parameters of the anterior (**A**) and posterior (**P**) intestine of Nile tilapia fed with oxytetracycline medicated feed for 10 days. Evaluation of the effects at day 10 (direct effect), and at days 11, 18, 25, and 40 (recovery). **C** (control diet), **O** (oxytetracycline diet), **OC** (oxytetracycline + control diet), **OP** (oxytetracycline + plant diet). Different **blue letters** mean significant differences among the dietary treatments in that intestinal portion ($p < 0.05$). The **orange letters** represent significant differences between the intestinal portions in that diet ($p < 0.05$).

During the recovery period, the dietary treatments induced some significant modifications on the intestinal wall, at days 18, 25 and 40 (highlighted by blue letters), and

some differences were also seen between the intestinal portions (represented by orange letters) (Figure 9).

4. Discussion

The gastrointestinal tract of fish is a multifunctional organ, displaying a complex defence mechanism against pathogens and a variety of physiological functions that are critical for nutrient digestion, immunity, and interaction with the environment (Salinas & Parra, 2015; Limbu et al., 2018; Almeida et al., 2019; Kumar et al., 2020). Intestinal morphology, associated immune system, and microbial community are closely related and dependent on each other (Kumar et al., 2020). Diets can be used to manipulate the microbial community and the intestinal structure, however, the microbiota stability and intestinal integrity has to be assured, not to induce intestinal inflammation and necrosis, and to prevent the occurrence of dysbiosis (Navarrete et al., 2008; Salinas & Parra, 2015). Antibiotics are frequently used in aquaculture to control diseases; however, when applied through diet, they have been associated with intestinal toxicity and dysbiosis (Navarrete et al., 2008, El-Adawy et al., 2018; Limbu et al., 2018; Almeida et al., 2019; Julinta et al., 2019). Plant ingredients are also being used in aquaculture as alternatives to fish meal and fish oil; however, imbalanced nutrient profiles, high amounts of fibre, and the presence of anti-nutritional factors may impair their use (Hassaan et al, 2018).

Aeromonas hydrophila and *Vibrio anguillarum* are some of the causative agents of bacterial diseases in Nile tilapia (FAO, 2005-2021), and since OTC is recommended to treat *Aeromonas* and *Vibrio* infections (DGAV, 2020), it was expected a decline of these bacterial populations. In fact, *Vibrio* counts were reduced after the oral treatment with OTC in this study, however, total culturable bacterial counts and *Aeromonas* counts did not decrease as found in previous studies (Islam et al., 2015; El-Adawy et al., 2018; Mannan et al., 2020). Even though fish intestinal microbial communities are very variable and highly affected by host factors (fish species, age, genetics) and external factors (surrounding environment, diet) (Navarrete et al., 2008; Sekkin & Kum, 2011; Salinas & Parra, 2015; Almeida et al., 2019; Kim et al., 2019; Abraham et al., 2020; Kumar et al., 2020), total culturable bacteria counts were not affected by the recovery diets in the present study. On the other hand, the anterior intestine of Nile tilapia in this study presented usually less culturable bacteria than the posterior, as also described by Cantas et al. (2012) on zebrafish.

Valdes et al. (2018) highlighted the importance of maintaining a diverse microbial community, rich in innocuous and beneficial bacteria, to assure good productivity and to prevent the proliferation or invasion of opportunistic microorganisms. In Nile tilapia, the normal microbiota has been associated with the presence of *Proteobacteria*, *Firmicutes*,

and *Actinobacteria* phyla (Bereded et al., 2020; Hossain et al., 2021; Serag et al., 2022); however, as previously described, very prone to modification to internal or external factors. Overall, intestinal microbiota indices were not significantly affected by the antibiotic or the recovery diets in this study; however, NGS analysis revealed a shift in the microbial community to *Proteobacteria*, *Fusobacteria*, and *Firmicutes* phyla in the anterior intestine, and to *Bacteroidetes*, *Verrucomicrobia*, and *Fusobacteria* phyla in the posterior intestine, after the ingestion of OTC. In fish, OTC has been referenced to lower the intestinal microbiota community indices (Navarrete et al., 2008; Payne et al., 2021), modify or eradicate OTUs (Limbu et al., 2018), and induce the presence of dominant phylotypes (Navarrete et al., 2008). Regarding *Fusobacteria*, this phylum is highly conserved in fish species and, as verified in this study, very resilient to OTC treatment (Zhou et al., 2018; Li et al., 2020; Payne et al., 2021). Additionally, fish species, culture conditions, antibiotic dose, or route of administration seem to influence the health effects produced by OTC treatments in fish (Grondel et al., 1987; Björklund & Bylund, 1990; Björklund et al., 1990; Yang et al., 2020).

The response at genera level to the antibiotic in this study was very distinct between the two intestinal portions. This variation was also verified by researchers in other fish species (Amillano-Cisneros et al., 2022) and can be related to the complex morphology, topography and physiology found in the intestinal tract of tilapias (Smith et al., 2000; Okuthe & Bhomela, 2020). Additionally, pH has been described to vary between the different intestinal portions of fish (Martínez Palacios et al., 2002), which imposes selective pressures on intestinal microbial communities and their metabolisms (Ilhan et al., 2017). It is important to emphasize that among the genera affected by the dietary treatment with OTC, some are normally found in freshwater environments (Mondino et al., 2020) and are autochthonous in Nile tilapia (Hossain et al., 2021), and others are related with probiotic properties (Picard et al., 2005). Despite the presence of potential fish pathogens, such as *Delftia acidovorans* (Andree et al., 2013) in the O group, no signs of tilapia disease were verified during the trial. It is also important to remember that some bacteria, such as *Clostridium* members, can operate as mutualistic symbionts, especially with herbivorous fish (Clements et al., 2007; Clements et al., 2009; Serag et al., 2022).

In this study, different diets were used to evaluate the recovery from the medicated treatment with OTC. The control diet, with fish meal, that fulfilled the known nutritional requirements for Nile tilapia, and the high plant-based diet, with the absence of fish meal and higher levels of high-fiber vegetable ingredients, such as full-fat soybean meal and sunflower meal. As previously described, no major changes were found in the recovery period, except for the increase of richness of the OP group on day 25. Previous studies highlighted that herbivorous and omnivorous fish presented more bacterial diversity than

carnivorous ones (He et al., 2013; Romero et al., 2014; Ye et al., 2014; Liu et al., 2016) which might explain the increased numbers of richness and diversity found in the OP group.

On the other hand, the dynamics of the microbial community in the intestine of Nile tilapia underwent some fluctuations in the recovery period. As referenced before by several authors (Navarrete et al., 2008; Sekkin & Kum, 2011; Salinas & Parra, 2015; Almeida et al., 2019; Kim et al., 2019; Abraham et al., 2020; Kumar et al., 2020), various aspects can affect the dynamic of tilapia´s intestinal microbiota. Despite these fluctuations, at the end of the trial (day 40), the recovery diets induced a unique bacterial population different from that found after the OTC treatment (day 10), with the neutralization of at least 45% of the antibiotic-induced variations of taxa. Also, the antibiotic-affected genera returned to normal values with the recovery diets, and the pathogenic-associated bacteria exclusively found in the O group did not emerge as unique in the OC and OP groups. Maybe in Nile tilapia, as in humans, although constantly exposed to internal and external challenges, the gut microbiota is very resilient, oscillating around a dynamic equilibrium, and is able to restore its equilibrium or to induce a new equilibrium state, after the cessation of the external perturbation (Fassarella et al., 2020). This new equilibrium after the antibiotic dietary treatment seems to be diet-dependent, as the recovery diets display an exclusive bacterial community, especially in the posterior intestine. Therefore, diet seems to play an important role in shaping the microbial community in fish species, as the gut microbiota, metabolic capacity and intestinal enzyme activity are influenced by the trophic level (Liu et al., 2016). The intestinal resident bacteria can metabolize protein and uses the dietary protein as a primary source of amino acids for protein synthesis and metabolic energy. These digestive products can be used by the microbes or be absorbed by the intestinal enterocytes in a mutualist relationship between the host and the microbiota community (Zhao et al., 2019). As plant or animal protein sources display a unique amino acid profile, the gut microbiota can be altered by the dietary protein quality and influences the host metabolism, regulating the intestinal barrier function, gut motility and the immune system (Zhao et al., 2019).

The intestinal tract of Nile tilapias follows a very complex morphology, related to their feeding habit (omnivorous), which provides a sufficient surface area to digest and absorb nutrients, allowing them to adapt to different diets (Smith et al., 2000). Although antibiotics are known to disrupt the tight junction proteins through oxidative stress damage, impairing the intestinal barrier functions and interfering in metabolic and immune-related metabolisms (Limbu et al., 2018), in this study, no significant changes were observed after the dietary treatment with OTC; however, in fish, OTC has been associated with decreased villi width and muscularis thickness (Limbu et al., 2018), and degeneration of epithelial layer, loss of absorptive vacuoles, necrotized intestinal villi, mucinous degeneration, and necrotized absorptive region (Julinta et al., 2019). Higher concentrations of drugs or longer periods of

treatment, especially above the permissible limits, seem to be necessary to induce severe toxic effects on the intestinal wall (Julinta et al., 2019).

Though high plant protein-based diets can induce intestinal inflammation in fish (especially in carnivorous) (Estruch et al., 2018; Kumar et al., 2020), no major changes were verified in OP group at the end of the trial. However, the posterior intestine was characterized by shorter intestinal villi height and area than the anterior portion, as previously described by Smith et al., (2000) and Okuthe & Bhomela, (2020) regarding Nile tilapia and Banded tilapia (*Tilapia sparrmanii*), respectively. Nile tilapia is an omnivorous species well adapted to plant ingredients (Ogello et al., 2014; Kuli Khan et al., 2013; Estruch et al., 2018; Hassaan et al., 2018; FAO, 2005-2021), with a wide range of hardiness and adaptability to adverse culture systems (FAO, 2005-2021). Several plant protein sources have been used successfully in feed formulations to replace fish meal (Al-Kenawy et al., 2008; Kuli Khan et al., 2013; Ogello et al., 2014). Blending different vegetable protein ingredients, feed processing and the inclusion of fed additives seems to help to overcome the limitations on the nutritional quality, bioavailability, and digestibility of vegetable ingredients, and to mitigate the effects of the anti-nutritional factors (Borlongan et al., 2002; Ogello et al., 2014; Hassaan et al., 2018). Still, it is important to remind that other types of diets (high-fat or high-carbohydrate diets), can worsen or attenuate the adverse effects caused by OTC in Nile tilapia (Limbu, et al., 2019; Limbu, et al., 2020), and it is of extreme importance to restore the intestinal equilibrium after the antibiotic therapy not to induce an unhealthy microbiota state responsible for chronic microbiota-related diseases (Fassarella et al., 2020).

5. Conclusions

The recommended dose in Portugal for the dietary treatment with OTC (55 mg Kg^{-1} body weight day^{-1}, 10 days) in fish undergoing bacterial diseases, originated in this study mild changes in the gut microbial community and the structure of the intestinal wall. The recovery diets helped to restore the OTC effects, and despite the aggressiveness of the high plant-based diet, the gut microbiota was able to recover. Nile tilapia presents a huge adaptability and hardiness to adverse culture practices and was able to endure even with a diet where fishmeal was completely replaced by vegetable ingredients.

6. Acknowledgments

This work was partially supported by Projects FICA (reference ALG-01-0247-FEDER-047175), UIDB/04423/2020 and UIDP/04423/2020, financed by Portugal and the European

Union through FEDER, COMPETE 2020, and CRESC Algarve 2020, in the framework of PORTUGAL2020, and through the COMPETE and Operational Human Potential Programs and national funds through Fundação para a Ciência e a Tecnologia (FCT, Portugal). This study was also partially funded by project ATLANTIDA (ref. NORTE-01-0145-FEDER-000040), supported by the North Portugal Regional Operational Program (NORTE2020), under the PORTUGAL2020 Partnership Agreement and through the European Regional Development Fund (ERDF). C. Teixeira and B. Costas were supported by FCT, Portugal (PD/BDE/135541/2018 and 2020.00290.CEECIND, respectively). FCT is also greatly acknowledged for the scientific employment contract of C.R. Serra. C. Teixeira was also supported by SPAROS through grant PD/BDE/135541/2018. We thank Fátima Faria (ICBAS) for monitoring the histological processing.

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Chapter IV

Dietary strategies to modulate the health condition and immune responses in gilthead seabream (*Sparus aurata*) juveniles following intestinal inflammation

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Abstract

Several feed additives have proved to be beneficial in eliciting fish health. β-glucans and curcumin are compounds with immunomodulatory capacities known to increase growth performance, stimulate immunity, improve general health, and enhance disease resistance in fish. The present study aimed to evaluate the effects of dietary *Phaeodactylum tricornutum* extracts rich in β-glucans and curcumin on gilthead seabream health status prior and following an intestinal inflammatory stimulus. Three experimental diets were formulated: a practical commercial-type diet (CTRL), a CTRL diet supplemented with 1% microalgae-derived β-glucans extract (BG), and a CTRL diet supplemented with 0.2% of curcumin (CUR). After 30 days of feeding trial, fish were sampled and subjected to an oral administration of 1% dextran sodium sulphate (DSS) to induce intestinal inflammation. Four groups were considered, while a group of fish continued to be fed on the CTRL diet, the remaining groups were exposed to DSS: CTRL-D (CTRL + DSS), BG-D (BG + DSS), and CUR-D (CUR + DSS) for 6 days. Growth, plasma and gut humoral immunity, liver and gut oxidative stress biomarkers, as well as intestinal gene expression were evaluated. No significant differences were found in growth after 30 days of feeding, however, seabream fed BG decreased anti-protease activity and nitric oxide concentration in plasma, while those fed CUR increased the mRNA levels of *tnfα*, *csf1r* and *hep* genes, compared to those fed CTRL. After the inflammatory stimulus, haematocrit was enhanced in fish fed BG-D and CUR-D, while red blood cells counts increased in those fed CTRL-D. Superoxide dismutase activity decreased in the intestine of all DSS groups while lipid peroxidation increased in the gut of fish fed CTRL-D and BG-D compared to CTRL. Moreover, mRNA expression levels of *csfr1* and *sod* decreased in fish fed CTRL-D and BG-D compared to CTRL, respectively. Despite the mild intestinal inflammatory condition induced by the DSS, CUR was able to partially ameliorate its effects, improving the haematological profile and assisting against the oxidative stress.

1. Introduction

The gilthead seabream (*Sparus aurata*) production has scaled up in the last years, due to its high demand and commercial value, translating into high stocking densities and stressful rearing conditions, which contributes to reduced growth, immunosuppression, higher fish mortality, and huge economic losses (Vetvicka et al., 2013; Ching et al., 2020; Ashry et al., 2021). Therefore, improving aquaculture practices, fish health and disease resistance of farmed fish is crucial (Meena et al., 2013). The demand of high productive aquaculture is pressuring the available supplies for aquafeeds formulations, and alternative ingredients are necessary to maintain the sustainability of this industry (Tacon & Metian, 2008; Naqtahnain Hamid et al., 2015). Unconventional alternatives to fish meal and fish oil are being extensively tested in aquaculture (Naqtahnain Hamid et al., 2015). However, lack of palatability, amino acid imbalance, poor digestibility, high amounts of fiber and non-starch polysaccharides, and anti-nutritional factors (saponins, alkaloids, tannins) can narrow their use in aquafeeds formulations (FAO, 2002). In addition, anti-nutritional factors can induce intestinal damage in fish and originate intestinal inflammation (enteritis), which reduce intestinal absorptive capacity, increase mucus secretion, induce hyperpermeability, enhance leucocyte infiltration, and activate the pro-inflammatory cytokines (TNFα, IL1β, IL8) (Estruch et al., 2018; Egerton et al., 2020; Kumar et al., 2020).

Dextran sodium sulphate (DSS) is a water-soluble sulphated polysaccharide detergent, with anti-coagulant proprieties, that creates a dysfunction in the intestinal barrier, with the production of toxic local effects, misbalance of the intestinal flora (dysbiosis), dissemination of the pro-inflammatory contents and dysregulation of the macrophage function (Chassaing et al., 2014; Jialing et al., 2020). Ingestion of DSS is a common method used to induce intestinal inflammation in several animal models (Arslan et al., 2007; Chassaing et al., 2014; Munyaka et al., 2016; Park et al., 2019; Jialing et al., 2020; Silvestri et al., 2020; Daskalaki et al., 2021; Xiang et al., 2021), including in fish (Oehlers et al., 2012; Oehlers et al., 2013; Wang et al., 2013; Marjoram et al., 2015; Morales Fenero et al., 2016; Hanyang et al., 2017; Lu et al., 2017; Chuang et al., 2019). DSS penetrates the mucosal membrane in the intestine and invades local macrophages, but it can reach other organs and tissues (Perse & Cerar, 2012).

A common practice to achieve economic sustainability and maximum production in aquaculture involves the use of functional feeds (Meena et al., 2013). A functional feed is a well-balanced diet supplemented with feed additives (Meena et al., 2013). Several feed additives (probiotics, prebiotics, nucleotides, phytochemicals) are known to be beneficial in improving fish health (Gannam & Schrock, 1999; Sakai, 1999; Ringo et al., 2011; Vallejos-Vidal et al., 2016; Dawood et al., 2018). Functional feeds can improve innate immunity and

disease resistance and are regarded as prophylactic alternatives to antibiotics and chemotherapeutics (Koch et al., 2021).

β-glucans are polysaccharides of glucose molecules, linked by either β-(1,3) or β-(1,4) liner backbone, with none, short or long β-(1,6) sidechain branches, that are usually originated from the cell wall of plants, fungi, bacteria, or seaweeds, and have different structures, sizes, solubility, activities, and immunomodulatory effects (Meena et al., 2013; Ching et al., 2020; Rodrigues et al., 2020). β-glucans can be used as immunostimulants, adjuvants or prebiotics and be administrated orally, intraperitoneal, or by immersion (Meena et al., 2013; Vetvicka et al., 2013; Rodrigues et al., 2020). Diatoms (*Bacillariophyta*) are photosynthetic eukaryotes found in aquatic environments (Granum & Myklestad, 2002). In diatoms, the carbohydrate content is mainly in the form of polysaccharides and divided into three groups, storage β-glucans (chrysolaminarin, water-soluble), cell wall polysaccharides (glucuronommannans, insoluble in water) and extracellular polysaccharides (chitin, water-soluble) (Granum & Myklestad, 2002). In *Phaeodactylum tricornutum*, the main β-glucan reserve polysaccharide is chrysolaminarin (β-1,3-glucan with β-1,6-branching) (Granum & Myklestad, 2002), similar to laminarin though with a lack of a terminal mannitol residue (Chiovitti et al., 2004). Chrysolaminarin is a low molecular weight soluble product of the photosynthesis process that is stored in the cell vacuoles (Granum & Myklestad, 2002; Chiovitti et al., 2004; Carballo et al., 2018). Chrysolaminarin-enriched extracts present a strong antioxidant activity and immunomodulatory effects in fish (Carballo et al., 2018), including in the gilthead seabream (Reis et al., 2021), and have been associated with higher survival rates and higher average weight in fish larvae (Skjermo et al., 2006).

Curcumin is a phytochemical compound obtained from the rhizomes of the turmeric herb (*Curcuma longa*), known to have biological properties, such as antioxidant, anti-inflammatory, antimicrobial, anti-cancer, and immunostimulant properties, and is also involved in growth promotion and increased disease resistance in fish (Ali et al., 2006; Sahu et al., 2008; Behera et al., 2011; Elgendy et al., 2016; Jiang et al. 2016; Mahmoud et al., 2017; Baldissera et al., 2018; Yonar et al., 2019; Li et al., 2020; Zheng et al., 2020; Mohamed et al., 2020, Alagawany et al., 2021). After ingestion, curcumin is transformed in metabolites and, about 75% of orally administered curcumin is excreted in faeces (Ali et al., 2006). The turmeric herb is rich in phenolic compounds (curcuminoids) known to be strong antioxidants (Ali et al., 2006).

DSS can be used in fish as an inflammatory model to study intestinal inflammation. The present study aimed to produce a controlled inflammatory process to evaluate the effects of two feed additives (*Phaeodactylum* β-glucans and curcumin) on gilthead seabream health condition following an intestinal inflammatory stimulus. Several biomarkers related to growth, immunity, oxidative stress and gene expression were assessed to evaluate the inflammatory

effects produced by the DSS as well as the potential modulatory proprieties of the feed additives on intestinal and health-related parameters.

2. Material and methods

2.1. Experimental diets

Three diets were formulated to be isonitrogenous (46% crude protein) and isolipidic (18% crude fat) (Table 1). A practical commercial-type diet was used as control (CTRL) whereas two experimental diets based on CTRL were supplemented with either a 0.2% of curcumin extract (CUR) or 1% of an extract of *P. tricornutum* rich in β-glucans (BG). Chrysolaminarin-rich biomass from *P. tricornutum* (SAG 1090 1b) was grown under nitrogen-depleted conditions in flat panel airlift reactors and was harvested and concentrated via centrifugation to 250-270 g L^{-1} (Clara 20, Alfa Laval) and frozen at -20°C. Afterwards, the biomass was thawed and diluted to 100 g L^{-1} with deionized water, and cells were disrupted with a ball mill (PML-2, Bühler). The disrupted biomass was then centrifuged, and the β-glucans rich supernatant was freeze-dried (Avanti J-26 XP, Beckman Coulter). The β-glucans content in this algae extract was 37%.

Diets were manufactured by extrusion at SPAROS LDA (Olhão, Portugal). All powder ingredients were mixed accordingly to the target formulation in a double-helix mixer (model 500L, TGC Extrusion, France) and ground (below 400 µm) in a micropulverizer hammer mill (model SH1, Hosokawa-Alpine, Germany). Diets (pellet size: 3.0 mm) were manufactured with a twin-screw extruder (model BC45, CLEXTRAL, France) with a screw diameter of 55.5 mm. Extruded pellets were dried in a vibrating fluid bed dryer (model DR100, TGC Extrusion, France). After cooling, oils were added by vacuum coating (model PG-10VCLAB, Dinnissen, The Netherlands). A second set of all diets was also made with the incorporation of 1% DSS (MW:40 KDa; TdB Labs AB, Sweden) by top-coating. Diets were stored in a temperature-controlled room.

Table 1: Ingredient and chemical composition of the diets (feeding trial).

Ingredients (%)*	Control diet	β-glucans diet	Curcumin diet
Fishmeal super prime	13.00	13.00	13.00
Fishmeal 60	7.00	7.00	7.00
CPSP 90	2.00	2.00	2.00
Poultry meal 65	15.00	15.00	15.00
Soy protein concentrate	5.00	5.00	5.00
Wheat gluten	4.20	4.20	4.20
Corn gluten meal	8.00	8.00	8.00
Soybean meal 48	10.00	10.00	10.00
Rapeseed meal	5.00	5.00	5.00
Sunflower meal	5.00	5.00	5.00
Wheat meal	11.13	10.13	10.13
Vitamin and mineral premix	1.00	1.00	1.00
Vitamin C35	0.05	0.05	0.05
Vitamin E50	0.02	0.02	0.02
Betaine HCl	0.20	0.20	0.20
Antioxidant	0.20	0.20	0.20
Fish oil	3.96	3.96	3.96
Soybean oil	5.28	5.28	5.28
Rapeseed oil	3.96	3.96	3.96
Curcumin extract 95%			0.20
Algae beta-glucans extract		1.00	
Proximate Analyses (% dry matter)			
Moisture	7.07		
Crude protein	46.16		
Crude fat	17.80		
Ash	8.34		
Gross energy (MJ Kg^{-1})	21.22		

***Fishmeal super prime:** 66.3 % CP, 11.5 % CF, Pesquera Diamante, Peru; **Fishmeal 60:** CONRESA 60 - 65 % CP, 10 % CF, Conserveros Reunidos S.A., Spain; **CPSP 90:** 86 % CP, 6 % CF, Sopropêche, France; **Poultry meal 65:** 65 % CP, 12 % CF, SAVINOR UTS, Portugal; **Soy protein concentrate:** Soycomil P - 62 % CP, 0.5 % CF, ADM Portugal, Portugal; **Wheat gluten:** VITEN - 81 % CP, 2.1 % CF, Roquette, France; **Corn gluten meal:** 58 % CP, 4 % CF, MPS, France; **Soybean meal 48:** solvent extracted soybean meal - 43 % CP, 2.7 % CF, CARGILL, Spain; **Rapeseed meal:** defatted rapeseed meal - 32.7 % CP, 4.1 % CF, Ribeiro & Sousa Lda, Portugal; **Sunflower meal 40:** solvent extracted dehulled sunflower meal - 43 % CP, 3 % CF, MAZZOLENI SPA, Italy; **Wheat meal:** 10.2 % CP; 1.2 % CF, MOLISUR, Spain; Vitamin and mineral premix **:** PREMIX Lda, Portugal - Vitamins (IU or mg kg^{-1} diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 500 mg; inositol, 500 mg; biotin, 3 mg; calcium pantothenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg kg^{-1} diet): copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulfate, 7.5 mg; sodium chloride, 400 mg; excipient wheat middling's; **Vitamin C35:** ROVIMIX STAY-C 35, DSM Nutritional Products, Switzerland; **Vitamin E50:** ROVIMIX E50, DSM Nutritional Products, Switzerland; **Betaine HCl:** ORFFA, The Netherlands; **Antioxidant:** VERDILOX PX, KEMIN EUROPE NV, Belgium; **Fish oil:** Sopropêche, France; **Soybean oil:** J.C. Coimbra, Portugal; **Rapeseed oil:** J.C. Coimbra, Portugal; **Curcumin extract 95 %:** Denk Ingredients (Munich, Germany); **Algae beta-glucans extract:** beta-glucans extract from microalgae (*Phaeodactylum tricornutum*).

2.2. Fish and experimental design

The experimental trial was executed at CIIMAR (Interdisciplinary Centre of Marine and Environmental Research) facilities (Matosinhos, Portugal). Gilthead seabream juveniles obtained from Sonríonansa, S.L. (Spain), weighing 34.0 ± 0.5 g, were randomly distributed in 12 tanks of 250 L of water capacity (25 fish each tank) and maintained in a seawater recirculating system. A photoperiod of 12 h light 12 h dark^{-1} was applied. Water dissolved oxygen was around 6.3 ± 0.5 mg L^{-1}, the water temperature was around 20.6 ± 0.5ºC, pH was around 7.7 ± 0.2, and salinity was around 35.3 ± 0.5 g L^{-1}. All animals were acclimatized to the experimental conditions for 10 days and fed with the CTRL diet twice a day, *ad libitum*.

After the acclimatization period, a feeding trial was performed, and three dietary treatments were randomly assigned. Fish from 6 tanks were fed with the CTRL diet, whereas fish from the other 6 tanks were fed with the BG or the CUR diet in triplicates. Seabream juveniles were fed twice a day, *ad libitum*, and the feeding trial lasted for 30 days (Figure 1).

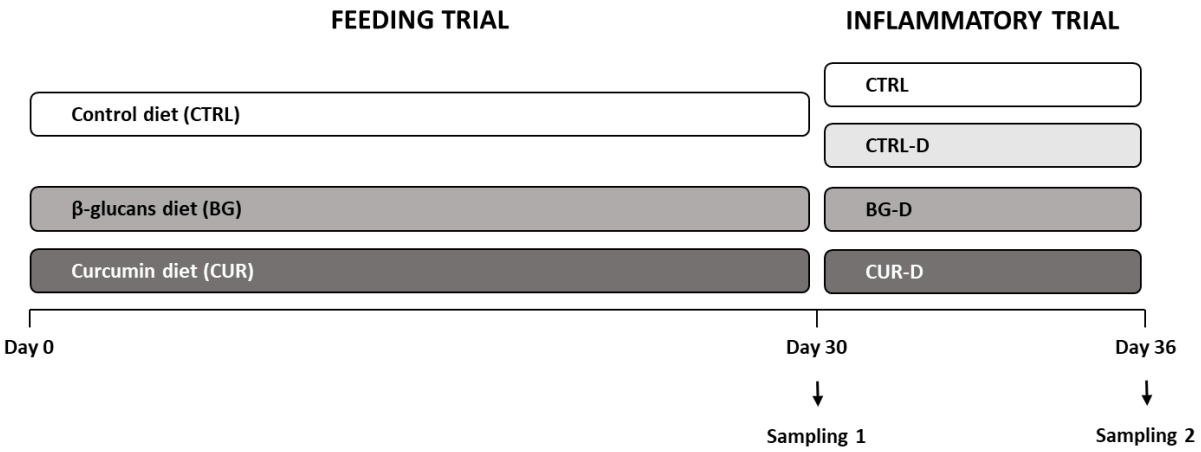

Figure 1. Experimental design of the feeding and gut inflammation trials. **CTRL** (control diet), **BG** (β-glucans diet), **CUR** (curcumin diet), **CTRL-D** (control diet + dextran sodium sulphate - DSS), **BG-D** (β-glucans diet + DSS), **CUR-D** (curcumin diet + DSS).

After the feeding trial, a dextran sodium sulphate (DSS) induced inflammation in the intestine was performed, using 1% of DSS in the diet. For this purpose, four dietary treatments were used in triplicates. Three tanks from the CTRL group remained with the CTRL diet, whereas the other 3 tanks shifted to the CTRL diet supplemented with DSS (CTRL-D). The BG and CUR groups were also fed with the BG and CUR diets supplemented with DSS (BG-D, CUR-D). The fish were fed twice a day, at a ration of 1.5% body weight day^{-1}, and the inflammatory trial lasted for 6 days (Figure 1).

All fish procedures followed Portuguese and European guidelines on the protection of animals used for scientific purposes, such as, “Directive 2010/63/UE” and “Decreto-Lei n. º 113/2013 de 7 de Agosto”, and FELASA category B and C recommendations.

2.3. Sample collection

At the end of the feeding and inflammatory trials (day 30 and 36, respectively), samples were taken to evaluate growth, health status, and inflammatory condition. Four fish per tank (n = 12) were randomly selected, anesthetized by immersion in 2-phenoxyethanol (1500 ppm; Sigma), and sampled for weight measurements, blood, and tissue. Blood was collected from the caudal vein using heparinized syringes (2500 U.I., Braun; 25 G, 1 mL). Then, the fish were opened, and the internal organs were placed in a petri dish. Liver samples were collected and stored at -80ºC, to evaluate oxidative stress. Several sections of 0.5 cm of the anterior intestine were collected, flushed with sodium phosphate buffer, and stored at -80ºC to evaluate immunity and oxidative stress or stored in RNAlater™ (Sigma) at -20 ºC for gene expression.

2.4. Haematological profile

The haematological profile was verified, and the haematocrit percentage (HT), total red blood cells (RBC) counts, total and differential white blood cells (WBC) counts were performed according to Machado et al. (2015). The haemoglobin (HG) content (Hemoglobin - Drabkin - colorimetric, Spinreact) was quantified. The mean corpuscular volume (MCV), the mean corpuscular haemoglobin (MCH) and the mean corpuscular haemoglobin concentration (MCHC) were calculated as described by Machado et al. (2015). From the homogenized blood, blood smears were prepared following Machado et al. (2015) protocol, and the identification of neutrophiles, through the presence of peroxidase activity, was carried out according to Afonso et al. (1998). Additionally, the blood smears were stained with Wright's stain (Haemacolor; Merck) to perform differential WBC counts, as described by Machado et al. (2015).

2.5. Plasma and intestinal humoral immune parameters

Immune-related parameters were analysed in plasma and intestine. The intestinal samples were homogenized (1:10) with K phosphate buffer (KPB) (K_2HPO_4 0.1 M, KH_2PO_4 0.1 M, pH 7.4, Sigma) using Precellys evolution tissue lyser homogenizer. The protease activity (%) in plasma was established as described by Ramos-Pinto et al. (2019) and calculated in comparison to the reference sample. The anti-protease activity (%) in plasma was determined according to Machado et al. (2015), however, the incubation with the phosphate buffer

(NaH2PO4, Sigma) and azocasein (Azocasein, Sigma) was performed for 1 h, at 22ºC, in the dark. The anti-protease activity inhibition was calculated in comparison to the reference sample. Total peroxidase activity in plasma and intestine (units mL^{-1}) was quantified following the protocol described by Machado et al. (2015), and calculated by defining one unit of peroxidase as that which produces an absorbance change of 1 OD. Total nitric oxide concentration (mg L^{-1}) in plasma and intestine was calculated using a colorimetric Nitrite/Nitrate Assay Kit (Roche Diagnostics GmbH), adapted for 96-well microplates, as described by Machado et al. (2015) and calculated based on the standard curve. Immunoglobulin M levels (Absorbance $OD_{450\ nm}$) in plasma and intestine were analysed by an Enzyme-linked Immunosorbent Assay (ELISA) according to Cuesta et al. (2004) and adapted by Ramos-Pinto et al. (2021), however we dilute 5 µL of plasma in 495 µL of sodium carbonate buffer (Na_2CO_3, 50 mM, pH = 9.6, Sigma). In the case of the intestine, 35 µL of homogenates were dissolved (1:10) in 315 µL of sodium carbonate (Na_2CO_3, 50 mM, pH = 9.6, Sigma), and the primary antibody was dissolved in a 1:200 proportion. All the analyses were conducted in triplicates.

2.6. Liver and intestinal oxidative stress

Liver and gut tissues were homogenized (1:10) with K phosphate buffer (K_2HPO_4 0.1 M, KH_2PO_4 0.1 M, pH 7.4, Sigma) using Precellys evolution tissue lyser homogenizer. Aliquots of 200 µL from homogenates were used to determine lipids peroxidation, and 4 µL of 4% 3,5-Di-tert-4-butylhydroxytoluene (BHT, in methanol, Sigma) was added to each sample, to prevent artefactual lipid oxidation, as described by Torres et al. (2002). The remaining homogenates were centrifuged at 10,000 × g for 20 min and 4°C, and the supernatant was stored at -80ºC until assayed.

The lipid peroxidation (nmol g^{-1}) was determinate with a microplate reader as adapted by Almeida et al. (2010), by measuring thiobarbituric acid-reactive substances (TBARS) as suggested by Bird and Draper (1984), with some adaptations performed by Peixoto et. al (2021). Briefly, the homogenates were incubated with 100 µL of TCA 100% and 1 mL of TBA 0.73%, Tris-HCL and DTPA (Sigma and Fluka) solution at 100°C for 60 min. The samples were then centrifuged at 11,500 x g for 5 min and 200 µL of the supernatant was added to the microplate wells. Absorbance was measured at 535 nm.

The colorimetric Pierce™ BCA Protein Assay Kit (Thermo Scientific™) was used to determine the total protein concentration (mg mL^{-1}), as described by Peixoto et al. (2021). Ten microliters of each sample were diluted (1:50) in 490 µL of K-phosphate buffer (0.1 M; pH 7.4) and 25 µL of each diluted sample was plated to microplate wells. Then, 200 µL of the reaction

buffer was added to each well and the absorbance was read at 562 nm in a Synergy HT microplate reader. Bovine serum albumin (BSA, 2 mg mL^{-1}) was used as standard.

Catalase activity (U mg^{-1} of protein) was determined by measuring the consumption of hydrogen peroxide (H_2O_2), as described by Clairborne (1985), adapted to the microplate reader by Almeida et al. (2010), and adjusted by Peixoto et al. (2021). Briefly, the samples were diluted to 0.7 mg mL^{-1} of protein in K-phosphate buffer (0.1 M; pH 7.4) and 10 µL of each diluted sample was plated in a UV microplate. After, 150 µL of the reaction buffer (K-phosphate buffer (0.05 M pH 7.0) and H2O2 30%) was added to each well and absorbance was read at 240 nm for 2 min (1 read every 15 seconds) in a Synergy HT microplate reader.

Total glutathione levels (nmol mg^{-1} of protein) were determined according to Griffith (1980), adapted to the microplate reader by Baker et al., (1990), with some modifications suggested by Peixoto et al. (2021). The samples were diluted to 0.7 mg mL^{-1} of protein in K-phosphate buffer (0.1 M; pH 7.4) and 50 µL of each diluted sample was plated to microplate wells. After, 250 µL of the reaction buffer (K-phosphate buffer (0.2 M, pH 8.0), NADPH (β-nicotanimide adenine dinucleotide 2′-phosphate reduced tetrasodium salt; Alpha Aesar), DTNB and glutathione reductase (Sigma)) was added to each well and absorbance was read at 412 nm for 3 min (1 read every 20 seconds) in a Synergy HT microplate reader.

Glutathione S-transferase activity (mU mg^{-1} of protein) was quantified by the conjugation of reduced glutathione with 1-chloro-2,4-dinitrobenzene (CDNB) according to Habig et al., 1974 and adapted to a microplate reader by Frasco & Guilhermino, 2002, with following the adaptions by Peixoto et al. (2021). The samples were diluted to 0.7 mg mL^{-1} of protein in K-phosphate buffer (0.1 M; pH 7.4) and 50 µL of each diluted sample was plated to microplate wells. After, 250 µL of the reaction buffer (K-phosphate buffer (0.2 M, pH 6.5), GSH, and CDNB solutions) was added to each well and absorbance was read at 340 nm for 5 min (1 read every 20 seconds) in a Synergy HT microplate reader.

Superoxide dismutase activity (U mg^{-1} of protein) was measured according to Flohe & Otting, 1984, adapted to the microplate reader by Lima et al. (2007), with some modifications. Briefly, the samples were adjusted to 0.3 mg mL^{-1} of protein concentration, with KPB and 50 µL of these suspensions were added to flat-bottomed 96-well plates. The KPB was used instead of liver or intestine homogenates as blank. Then, 200 µL of the reaction solution (0.064 mmol L^{-1} of xanthine in 1 mmol L^{-1} of NaOH and 0.0273 mmol L^{-1} cytochrome c in 50 mmol L^{-1} Na - phosphate buffer (pH 7,8) with 1 mmol L^{-1} Na-EDTA), and 50 µL of xanthine oxidase solution (0.03 U mL^{-1}) were added to each well. Final assay concentrations per well were 0.042 mmol L^{-1} of xanthine, 0.018 mmol L^{-1} cytochrome c, 0.005 U mL^{-1} xanthine oxidase solution, 0.06 mmol L^{-1} of NaOH, 30 mmol L^{-1} of NaH_2PO_4 and Na_2HPO_4 and 0.62 mmol L^{-1} Na-EDTA. The reaction was monitored by the formation of superoxide anion at 550 nm every 20 sec for

3 min on Synergy™ HT microplate reader (Bio-Tek®). Enzyme activity is expressed as enzyme units per milliliter of total protein (U mL^{-1} protein). All analyses were conducted in triplicates.

2.7. Intestinal gene expression

Anterior portions of the intestine were analysed for mRNA expressions to evaluate innate immunity, oxidative stress, inflammation, and DNA damage. Total RNA isolation the intestines were placed in a 2 mL tube containing 0.5 mL of Trizol (Nzol Reagent, NZYTech, Portugal) and two Zirconium Oxide-coated ceramic grinding sphere (2 mm diameter) and homogenised in a Precellys 24 tissues homogenizer (Bertin Ins., France) by 2 cycles of 6000 x g for 20 seconds. After, 150 µl of chloroform was added to the homogenate, gently vortexed and centrifuged for 15 min at 12000 x g and 4°C. Around 300 µl of the aqueous phase were transferred to a new 1.5 ml tube with 300 µl of 70% ethanol and gently homogenised. After this step, the RNA extraction was done with a NZY Total RNA Isolation Kit (NZYTech, Lisbon, Portugal) following the manufacturer's specifications. Quantification and purity of RNA were assessed by spectrophotometry and the 260:280 and 260:230 ratios were determined. RNA integrity was verified through 1.5% agarose gel electrophoresis. First-strand cDNA was synthesized with NZY First-Strand cDNA Synthesis Kit (NZYTech, Lisbon, Portugal) following the manufacturer's specifications. Primers were selected using Genebank and other publications and described in Table 2. Efficiencies were calculated in a serial five-fold dilutions of cDNA, using the slope of the regression line of the cycle thresholds (Ct) versus the relative concentration of cDNA as described by Machado et al. (2018). The amplification of primer dimers was also verified through melting curve analysis. Quantitative PCR assays were performed with an iQ5 Real Time PCR detection System (BioRad) using 4.4 µl of diluted cDNA (1:50 dilution) mixed with 5 µl of iQ SYBR green 2x Supermix (Bio-Rad) and 0.3 µl (10 mM) of each specific primer in a final volume of 10 µL. The standard cycling conditions were, initially one cycle of 95 ºC for 10 min, one cycle of 95ºC for 15 sec and one cycle of MT for 1 min, followed by 40 cycles of 95ºC for 15 sec, one cycle of 95ºC for 1 min and one cycle of the MT for 30 sec, followed by a melting curve from the MT to 95ºC, with increments of 0.5ºC for each 0.5 sec, and finally a cycle of 95ºC for 15 sec. All reactions were carried out as technical duplicates. The expression of the target gene was normalized using the expression of the 18S gene.

Table 2: Oligonucleotide primers information used for Real-time PCR analysis of gene expression.

Gene name	Symbol	Gene bank	Forward primer	Reverse primer	PL[1]	MT[2]	E[3]
Mn superoxide dismutase	*sod*	JQ308833	CCTGACCTGACCTACGACTATGG	AGTGCCTCCTGATATTTCTCCTCTG	134	60	2.05
Glutathione peroxidase 1	*gpx*	DQ524992	GAAGGTGGATGTGAATGGAAAAGATG	CTGACGGGACTCCAAATGATGG	129	60	2.21
Tumour necrosis factor alpha	*tnfα*	AJ413189.2	TGAACAGAGGCGACAAACTG	GCCACAAGCGTTATCTCCAT	245	60	1.92
Interleukin-10-like	*il10*	XM_30418889.1	AACATCCTGGGCTTCTATCTG	GTGTCCTCCGTCTCATCTG	65	57	2.07
Immunoglobulin M heavy chain	*igm*	AM493677	CAGCCTCGAGAAGTGGAAAC	GAGGTTGACCAGGTTGGTGT	136	59	2.19
Macrophage colony stimulating factor receptor	*csf1r*	AM050293	ACGTCTGGTCCTATGGCATC	AGTCTGGTTGGGACATCTGG	129	60	2.11
CD8 alpha chain precursor	*cd8α*	AJ878605	CTCGACTGGTCGGAGTTAA	TCCATCAGCGGCTGCTCGT	287	60	1.91
Heat shock protein 70	*hsp70*	DQ524995.1	ACGGCATCTTTGAGGTGAAG	TGGCTGATGTCCTTCTTGTG	124	55	2.05
Occludin	*ocln*	KF861990.1	TCATCTCCTACCCTCCAGTCA	ATGGTCTGCTTGTGGTCCTC	96	60	2.03
Hepcidin 1	*hep*	EF625901	GCCATCGTGCTCACCTTTAT	CCTGCTGCCATACCCCATCTT	382	60	2.07
Mucin-13-like	*muc13*	XM030399162	TTCAAACCCGTGTGGTCCAG	GCACAAGCAGACATAGTTCGGATAT	67	60	1.96
Mucin-2-like	*muc2*	XM_030425504.1	GTGTGTGGCTGTGTTCCTTGCTTTGT	GCGAACCAGTCTGGCTTGGACATCA	67	60	1.98

[1] Product length (Amplicon (nt))

[2] Melting temperature

[3] Efficiency of PCR reactions

2.8. Statistical analysis

All the data were expressed as means (M) and standard deviation (SD) and analysed for normality and homogeneity of variance. When required, results were log transformed before being treated statistically to ensure normality and homogeneity of data. Outliers were removed from the analysis. For each trial one-way ANOVA was used and the Tukey post hoc test was used to identify differences in the experimental treatments. All the statistical analysis was performed using IBM SPSS Statistics 24. The level of significance for all statistical tests used was $p < 0.05$.

3. Results

3.1. Growth performance

Growth performance was evaluated during the feeding and inflammatory trials. The feeding behaviour of the juvenile gilthead seabream did not change during the trials, and the entire feed rations were consumed. No statistical differences were found in the final body weight between the dietary treatments of both trials (Figure 2).

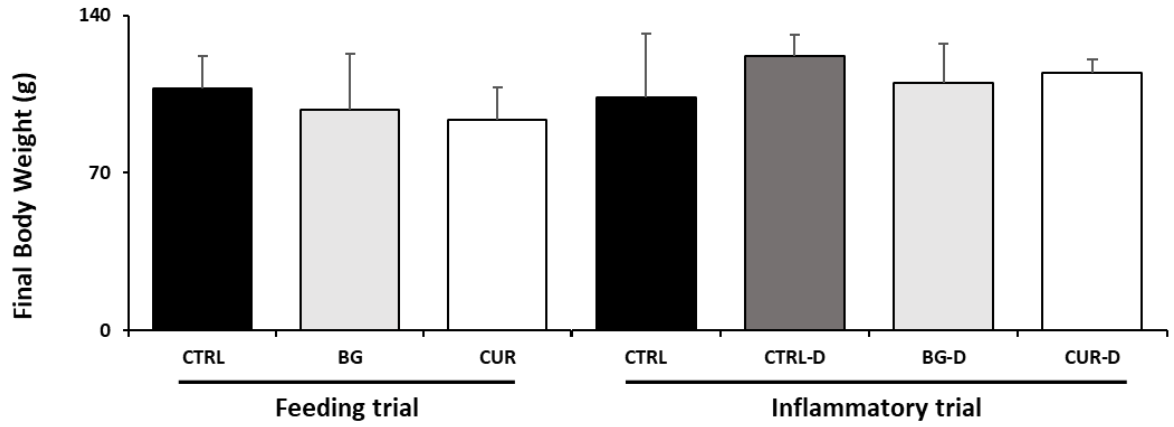

Figure 2. Final body weight of gilthead seabream after the feeding and inflammatory trials (n=12). **CTRL** (control diet)**, BG** (β-glucans diet)**, CUR** (curcumin diet), **CTRL-D** (control diet + dextran sodium sulphate - DSS), **BG-D** (β-glucans diet + DSS), **CUR-D** (curcumin diet + DSS).

3.2. Haematological profile

The effects of different dietary treatments in the haematological profile are described in Table 3. While no significant differences were found among dietary treatments at the end of the feeding trial, the DSS increased RBC and peripheral thrombocyte counts in fish fed CTRL-

D. In contrast, HG concentration dropped in fish fed CTRL-D compared to their counterparts fed CUR-D. A synergistic effect of DSS and dietary additives was observed for HT with increased values in fish fed BG-D and CUR-D compared to non-stimulated animals (i.e. CTRL). A similar effect was observed for peripheral lymphocytes in seabream fed BG-D compared to unstimulated fish fed CTRL. Moreover, MCV decreased in seabream fed CTRL-D compared to CTRL while no changes were observed in those fish fed BG-D and CUR-D dietary treatments. Fish fed BG-D also augmented MCH compared to those fish fed CTRL-D.

Table 3: Haematological profile of gilthead seabream after the feeding and inflammatory trials (n=12). **CTRL** (control diet)**, BG** (β-glucans diet)**, CUR** (curcumin diet), **CTRL-D** (control diet + dextran sodium sulphate - DSS), **BG-D** (β-glucans diet + DSS), **CUR-D** (curcumin diet + DSS). Values are presented as means ± standard deviation. Different letters mean significant differences among dietary treatments ($p < 0.05$).

Parameters*	Feeding trial			Inflammatory trial			
	CTRL	BG	CUR	CTRL	CTRL-D	BG-D	CUR-D
HT (%)	37.4 ± 6.7	36.4 ± 5.7	37.2 ± 6.9	37.0^{b} ± 6.4	39.7^{ab} ± 3.8	45.3^{a} ± 3.6	43.7^{a} ± 5.2
RBC (x 10^6 mL)	1.4 ± 0.5	1.3 ± 0.4	1.2 ± 0.6	2.0^{b} ± 0.9	2.9^{a} ± 0.8	2.3^{ab} ± 0.7	2.7^{ab} ± 0.1
HG (g dL^{-1})	2.4 ± 1.0	2.6 ± 1.2	2.0 ± 0.7	2.0^{ab} ± 0.3	1.8^{b} ± 0.6	2.3^{ab} ± 0.8	2.9^{a} ± 1.1
MCV (μm^3)	273.4 ± 72.8	310.07 ± 100.9	362.7 ± 209.6	209.7^{a} ± 70.9	134.7^{b} ± 27.1	190.1^{a} ± 48.3	162.1^{ab} ± 30.8
MCH (pg $cell^{-1}$)	15.0 ± 3.2	16.1 ± 5.2	13.2 ± 3.9	11.5^{ab} ± 4.8	7.0^{b} ± 2.8	12.8^{a} ± 4.3	10.5^{ab} ± 3.2
MCHC (g 100 ml^{-1})	6.6 ± 2.7	7.6 ± 4.0	4.8 ± 0.5	5.4 ± 1.4	4.9 ± 1.9	6.3 ± 1.1	6.6 ± 2.0
WBC (x 10^4 ml)	1.9 ± 0.9	2.0 ± 0.7	1.9 ± 0.7	1.7 ± 0.9	2.9 ± 1.7	3.0 ± 1.4	2.1 ± 1.3
Thrombocytes (x 10^4 ml)	0.9 ± 0.4	1.2 ± 0.5	1.0 ± 0.4	0.7^{b} ± 0.3	1.6^{a} ± 1.0	1.6^{a} ± 0.8	1.2^{ab} ± 0.7
Lymphocytes (x 10^4 ml)	0.6 ± 0.2	0.6 ± 0.2	0.6 ± 0.2	0.5^{b} ± 0.3	0.7^{ab} ± 0.3	1.0^{a} ± 0.5	0.6^{ab} ± 0.4
Monocytes (x 10^4 ml)	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.0
Neutrophils (x 10^4 ml)	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.2	0.1 ± 0.2

*__HT__ (haematocrit), **RBC** (red blood cells), **HG** (haemoglobin), **MCV** (mean corpuscular volume), **MCH** (mean corpuscular haemoglobin), **MCHC** (mean corpuscular haemoglobin concentration), **WBC** (white blood cells).

3.3. Plasma humoral immunological parameters

After the feeding trial, seabream fed BG dropped plasma anti-protease activity and nitric oxide levels compared to fish fed the CTRL diet, while fish fed CUR showed lower plasma peroxidase levels than seabream fed BG. After the gut inflammation trial, no significant differences were observed among the dietary treatments in the plasma humoral immunological parameters (Figure 3).

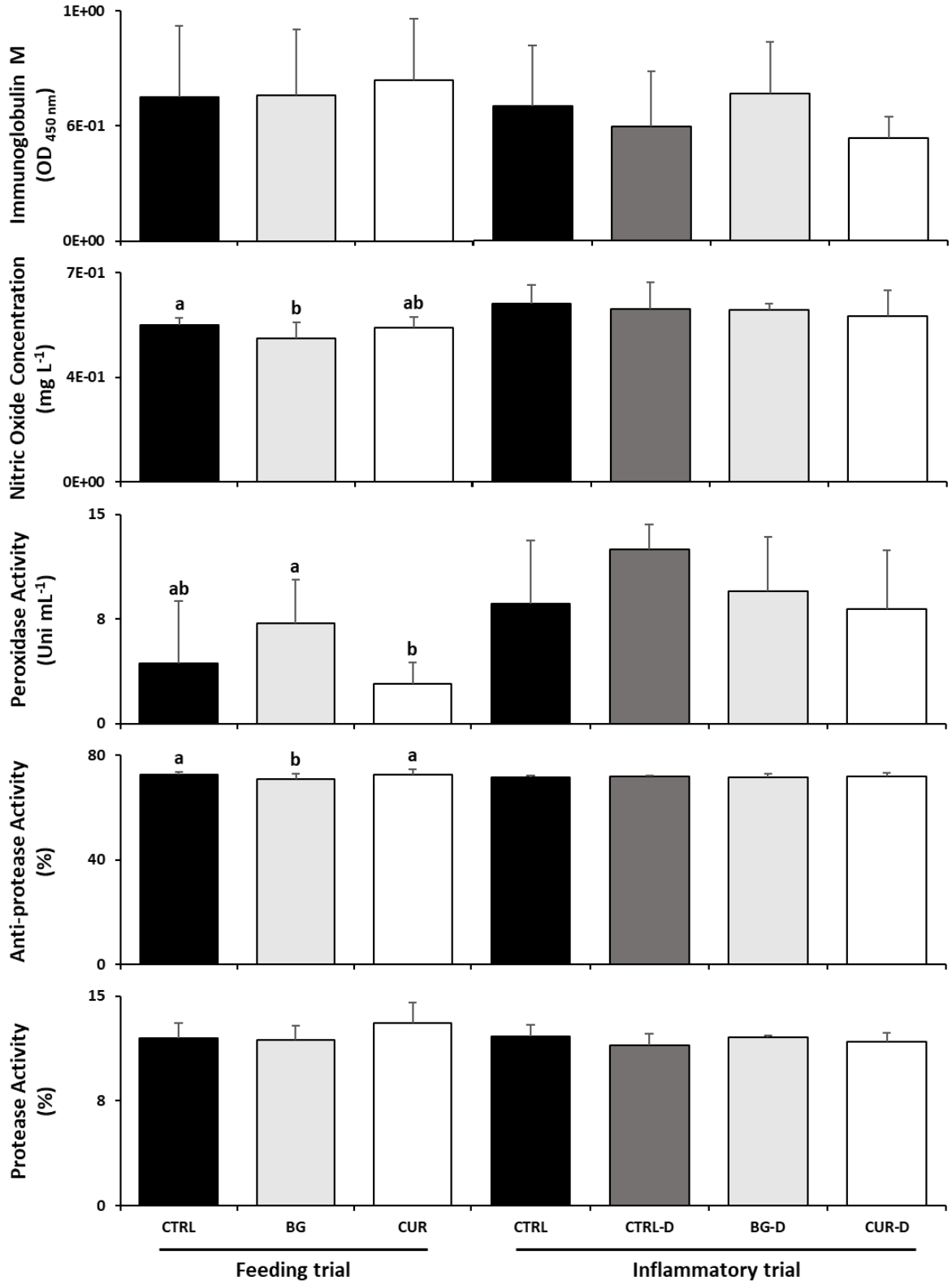

Figure 3. Humoral immunological parameters in plasma of gilthead seabream after the feeding and inflammatory trials (n=12). **CTRL** (control diet)**, BG** (β-glucans diet)**, CUR** (curcumin diet), **CTRL-D** (control diet + dextran sodium sulphate - DSS), **BG-D** (β-glucans diet + DSS), **CUR-D** (curcumin diet + DSS). Different letters mean significant differences among dietary treatments ($p < 0.05$).

3.4. Liver oxidative stress

As Figure 4 shows, there were no significant differences among fish fed the experimental diets and their counterparts fed the CTRL diet at the end of the feeding trial. However, fish fed CUR increased significantly the activity of hepatic catalase compared to seabream fed BG. Likewise, after the gut inflammation trial, there were no significant differences between the dietary treatments in the liver oxidative stress parameters.

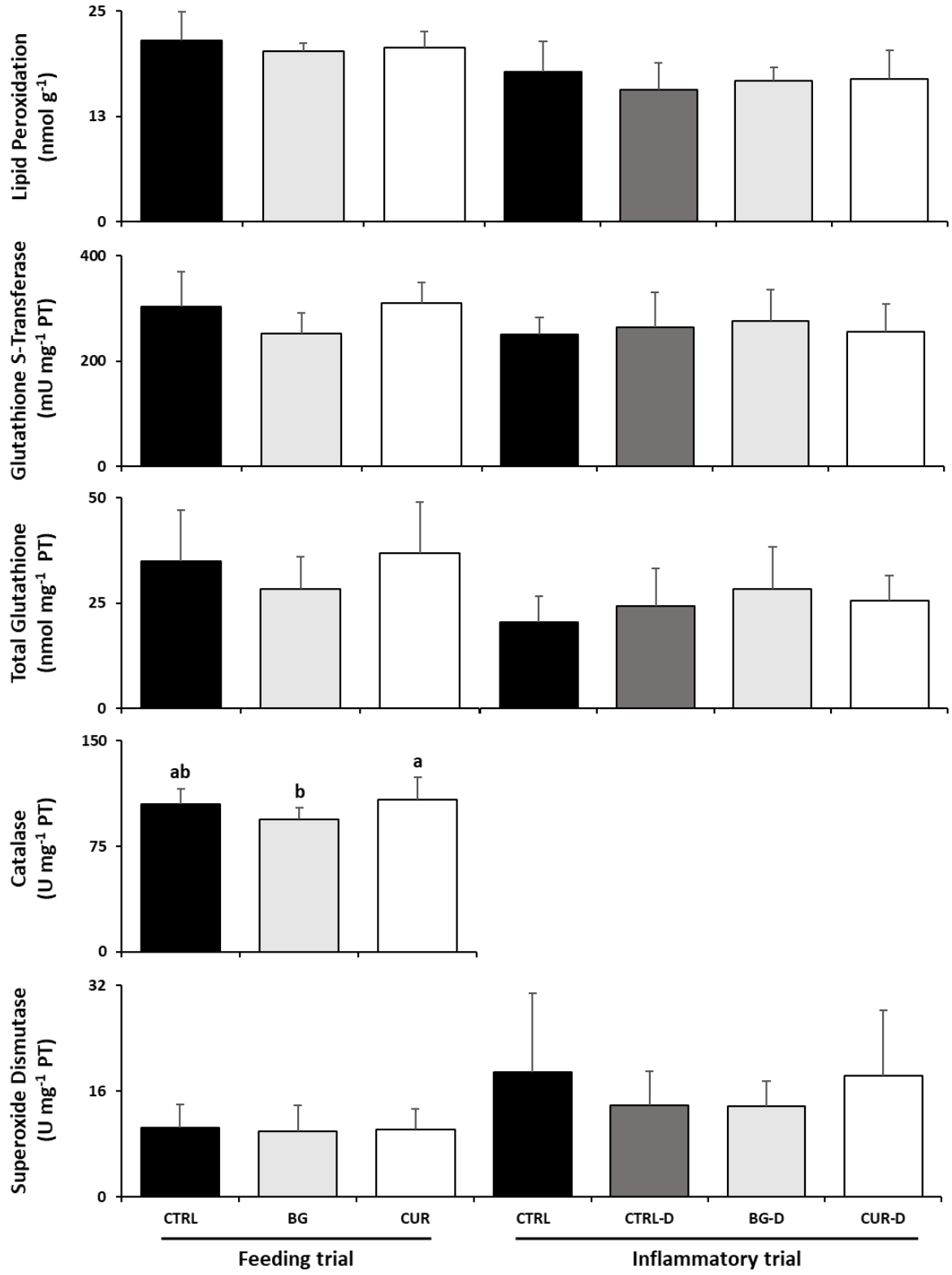

Figure 4. Liver oxidative stress biomarkers of gilthead seabream after the feeding and inflammatory trials (n=12). **CTRL** (control diet)**, BG** (β-glucans diet)**, CUR** (curcumin diet), **CTRL-D** (control diet + dextran sodium sulphate - DSS), **BG-D** (β-glucans diet + DSS), **CUR-D** (curcumin diet + DSS). Different letters mean significant differences among dietary treatments ($p < 0.05$).

3.5. Intestinal humoral immunological parameters

No significant differences were found in the intestinal humoral immunological parameters among seabream fed dietary treatments at the end of the feeding trial (Figure 5), although gut peroxidase activity and oxide nitric levels tended to drop in the fish fed the BG diet. Similarly, no changes were observed among dietary treatments after the gut inflammation trial.

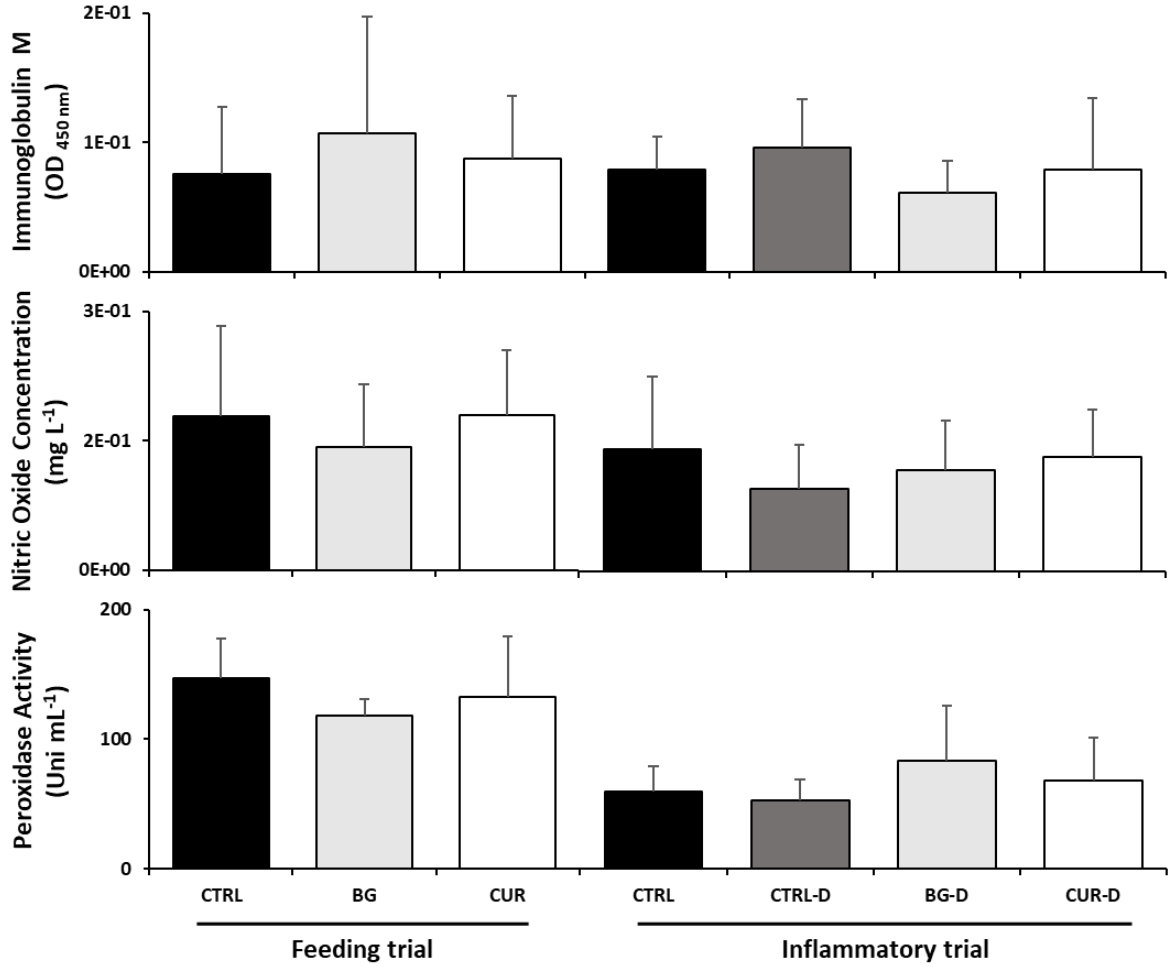

Figure 5. Intestinal humoral immunological parameters of gilthead seabream after the feeding and inflammatory trials (n=12). **CTRL** (control diet)**, BG** (β-glucans diet)**, CUR** (curcumin diet), **CTRL-D** (control diet + dextran sodium sulphate - DSS), **BG-D** (β-glucans diet + DSS), **CUR-D** (curcumin diet + DSS). Different letters mean significant differences among dietary treatments ($p < 0.05$).

3.6. Intestinal oxidative stress

Gilthead seabream were not affected by dietary treatments at the end of the feeding trial (Figure 6). However, the gut inflammation induced by DSS affected intestinal oxidative stress biomarkers, by decreasing superoxide dismutase activity in fish fed CTRL-D, BG-D and CUR-D compared to those fed CTRL. While lipid peroxidation levels increased in the gut of

seabream fed either CTRL-D or BG-D compared to CTRL, those levels dropped in fish fed CUR-D though not significantly different compared to their counterparts fed CTRL-D and BG-D.

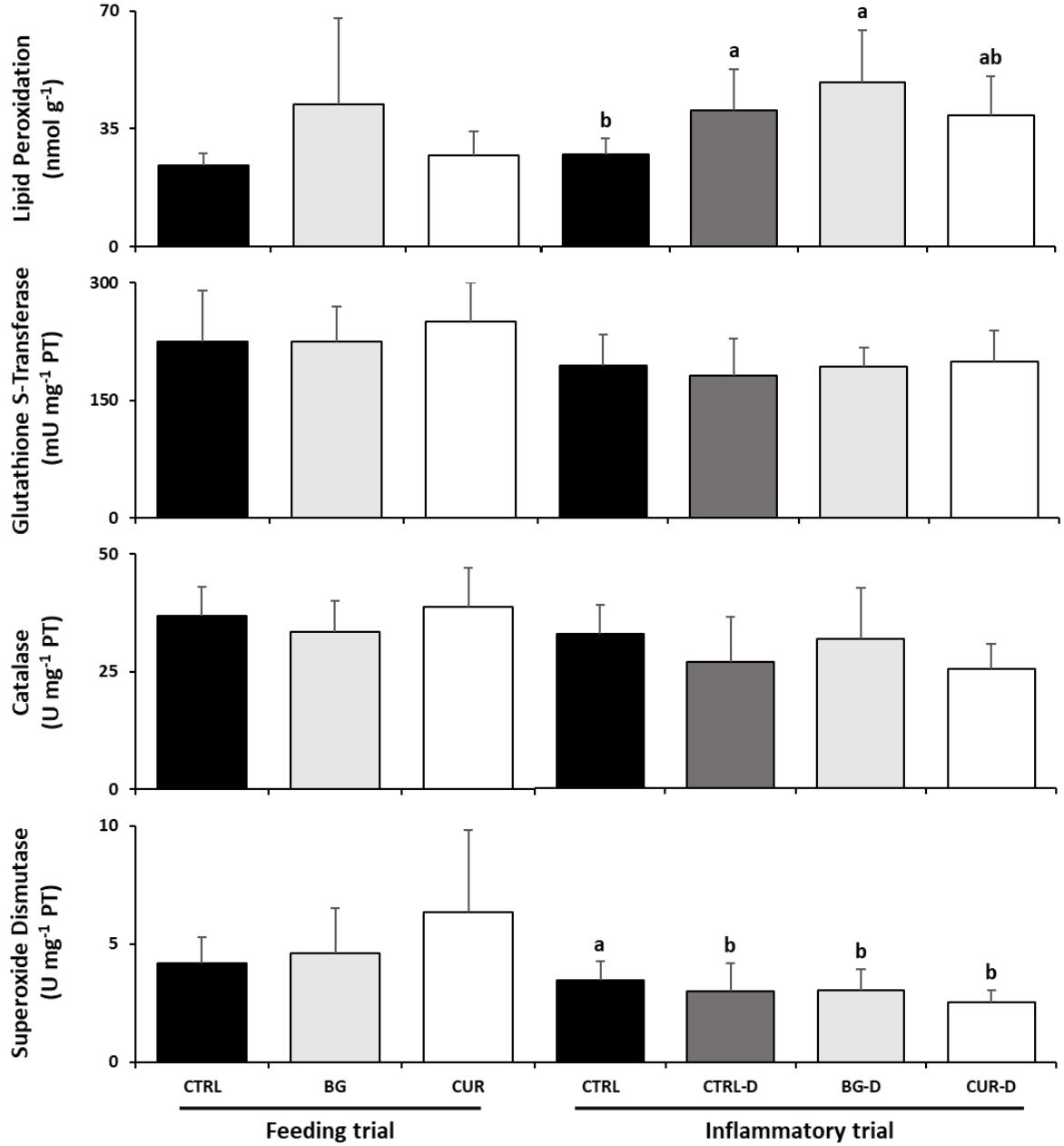

Figure 6. Intestinal oxidative stress biomarkers of gilthead seabream after the feeding and inflammatory trials (n=12). **CTRL** (control diet)**, BG** (β-glucans diet)**, CUR** (curcumin diet), **CTRL-D** (control diet + dextran sodium sulphate - DSS), **BG-D** (β-glucans diet + DSS), **CUR-D** (curcumin diet + DSS). Different letters mean significant differences among dietary treatments ($p < 0.05$).

3.7. Intestinal gene expression

The gene expression profiles in the intestine of gilthead seabream after both the feeding and the gut inflammation trials are described in Figure 7 and Supplementary Table 1. Fish fed CUR dietary treatment presented an upregulation of *tnfα, csf1r* and *hep* genes compared to their counterparts fed the CTRL diet. Following the gut inflammation trial, most target genes showed a tendency to be downregulated by the DSS treatment. However, only *csf1r* transcripts decreased significantly in fish fed CTRL-D compared to specimens fed CTRL. Moreover, *sod* mRNA expression also decreased significantly in seabream fed BG-D dietary treatment compared to fish fed CTRL (Figure 7).

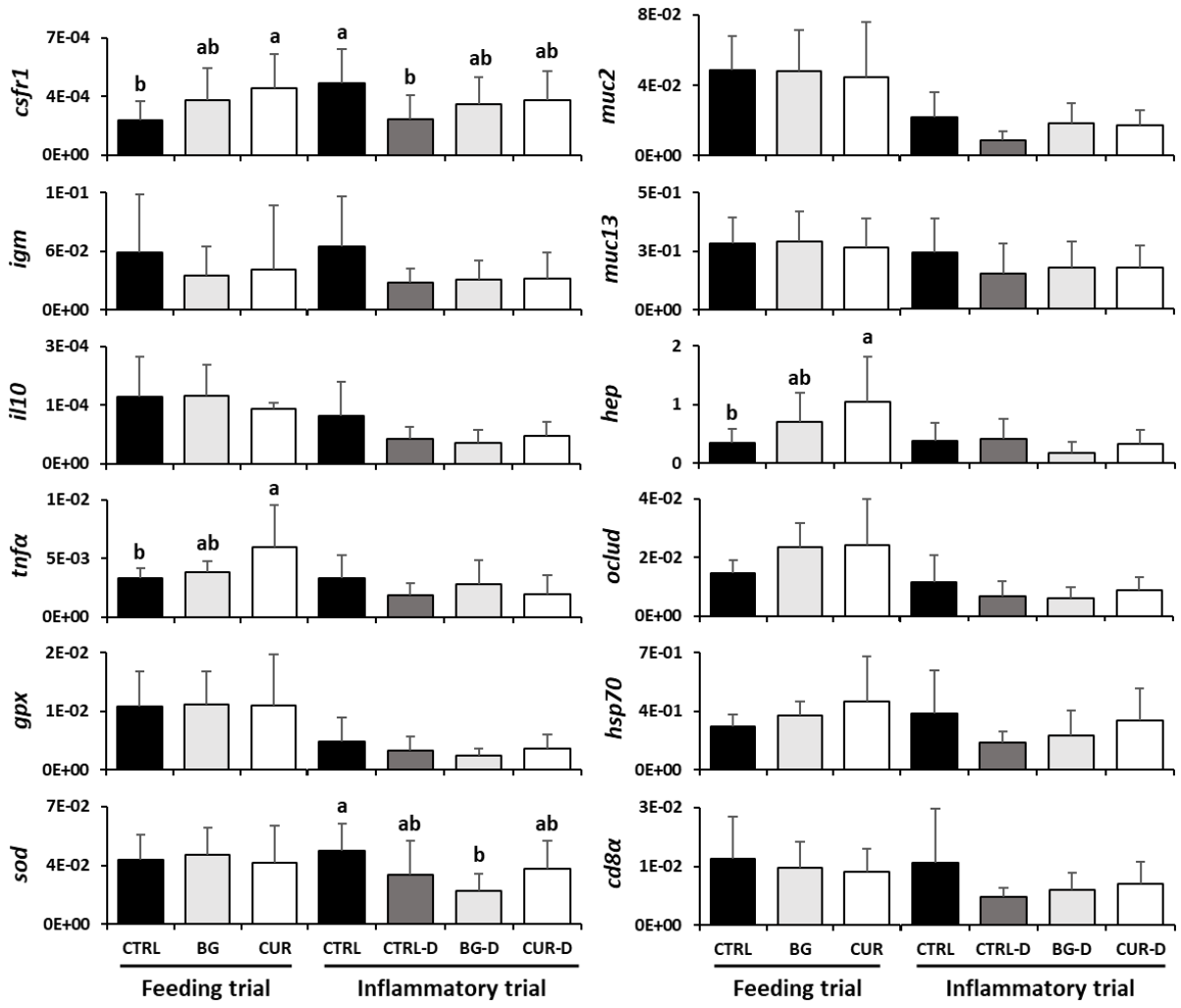

Figure 7. Intestinal gene expression profiles of gilthead seabream after the feeding and inflammatory trials (n=12). **CTRL** (control diet)**, BG** (β-glucans diet)**, CUR** (curcumin diet), **CTRL-D** (control diet + dextran sodium sulphate - DSS), **BG-D** (β-glucans diet + DSS), **CUR-D** (curcumin diet + DSS). Different letters mean significant differences among dietary treatments ($p < 0.05$).

4. Discussion

Preventing fish diseases and economic losses is crucial in order to increase profitability and sustainability of the aquaculture industry (Vetvicka et al., 2013; Ching et al., 2020; Ashry et al., 2021). The use of functional feeds can improve fish health and disease resistance, acting as prophylactic alternatives to antibiotics and others chemotherapeutics (Meena et al., 2011; Ringo et al., 2011). Some feed additives, such as β-glucans and curcumin, have multiple beneficial effects on fish, potentiating growth, physiological functions, immunity, antioxidant activity, and disease resistance (Ali et al., 2006; Meena et al., 2011; Ching et al., 2020; Mahmoud et al., 2017; Mohamed et al., 2020; Alagawany et al., 2021). In the present study, while some minor changes were observed at the systemic level in fish fed dietary treatments at the end of the feeding trial, health promoting effects were evident at the local level (i.e. gut) due to dietary curcumin supplementation. The increase in intestinal *tnfα, csf1r* and *hep* in fish fed CUR suggest activation of the innate immune response and mobilization of intraepithelial macrophages. Thought the response of fish to the dietary treatment with feed additives may not be ample in the absence of a stressor (Rodrigues et al., 2020), curcumin is known to have immunomodulatory properties in fish (Sahu et al., 2008; Behera et al., 2011; Elgendy et al., 2016; Mahmoud et al., 2017; Baldissera et al., 2018; Yonar et al., 2019).

Other biological property associated with curcumin and β-glucans are their anti-inflammatory capacity (Zhu et al., 2016; Alagawany et al., 2021). Therefore, this study also aimed to evaluate the ability of these two feed additives to minimize DSS inflammatory effects, since DSS is a common method used to study the intestinal inflammatory process in several animal models (Arslan et al., 2007; Chassaing et al., 2014; Munyaka et al., 2016; Park et al., 2019; Jialing et al., 2020; Silvestri et al., 2020; Daskalaki et al., 2021; Xiang et al., 2021), including in fish (Oehlers et al., 2012; Oehlers et al., 2013; Wang et al., 2013; Marjoram & Bagnat, 2015; Morales Fenero et al., 2016; Hanyang et al., 2017; Lu et al., 2017; Chuang et al., 2019), and feed additives can be used to reverse the effects of some intestinal inflammatory compounds (Nordvi et al., 2023).

Intestinal inflammation is a defensive process to control microbial infections and tissue damage (Burge et al., 2019). However, when dysregulated, intestinal inflammation involves a dysfunctional response of the host towards the diet, bacteria, and chemicals (Marjoram & Bagnat, 2015; Morales Fénero et al., 2016; Lu et al., 2017; Burge et al., 2019), with the overproduction of pro-inflammatory cytokines, such as TNFα, IL1β, and IFNγ, triggered by the activation of the NFκB pathway, that originates the collapse of the intestinal barrier (Hewlings & Kalman, 2017; Burge et al., 2019). According to previous studies in other animal models, intestinal inflammation induced by DSS is related to the overexpression of *tnfα, il-1β, il-6,* and *il-17* (Oehlers et al., 2012; Wang et al., 2013; Chassaing et al., 2014; Munyaka et al., 2016;

Park et al., 2019; Silvestri et al., 2020; Xiang et al., 2021). Nonetheless, in this study the expression of *tnfα* did not differ significantly among dietary treatments. The effectiveness of the DSS-induced inflammation depends on the concentration, duration, frequency, and molecular weight of the DSS administration, but also, the species and susceptibility of the animal model (Eichele & Kharbanda, 2017). In the present study, the dosage of DSS used did not appear to produce a significant extensive inflammation in the intestine of gilthead seabream. According to Oehlers et al. (2012) and Oehlers et al. (2013), an immersion with 0.5 % (w/v) DSS is sufficient to induce intestinal inflammation in zebrafish larvae, whereas an oral perfusion with 5 % DSS (Wang et al., 2013) can also be used in pufferfish.

In this study, a clear tendency to decrease intestinal *hsp70*, *muc2* and *IgM* transcripts may suggest that DSS can also induce harmful effects in the gut of seabream to some extent, a fact further corroborated by the significant increase in gut lipid peroxidation. The gastrointestinal tract has a protective mucus barrier, where mucins protect the surface of the intestinal epithelial cells (Eichele & Kharbanda, 2017; Hanyang et al., 2017). Mucin depletion, epithelial degeneration, necrosis, and inflammation are the major histologic features found in the intestine after the ingestion of DSS in mice and pigs (Perše & Cerar, 2012; Rattigan et al., 2020). Heat shock proteins (HSPs) are involved in the regulation of the intestinal immune functions (Hanyang et al., 2017), maintaining gastrointestinal homeostasis (Wang et al., 2018), and protecting the tissues from stressors (Akedemir et al., 2016). When HSPs levels are reduced, the severity of the intestinal inflammation is enhanced (Wang et al., 2018). In the present study, BG-D and CUR-D seemed to partially restore the drop in *muc2* and *hsp70* mRNA expression due DSS treatment, an interesting fact that should be further explored in future trials since there is potential in the use of these dietary treatments in seabream. Carballo et al., 2018 also verified an improved expression of *hsp90* in the kidney and spleen of sole, after intraperitoneal injections with chrysolaminarin. An increased expression of the *hsp70* gene and of the HSP70 protein was also found in rat neuronal cells treated with curcumin (Xia et al., 2015).

Curcumin is a powerful anti-inflammatory compound, that modulates many inflammatory mediators, decreasing the response of the NF-kB signalling pathway and, consequently, reducing the production of inflammatory cytokines (TNFα, IL1β, IL2, IL6, IL8, and IL12) (Ali et al., 2006; Cao et al., 2015; Hewlings & Kalman, 2017; Burge et al., 2019; Giri et al., 2019; Rajabiesterabadi et al., 2020). IL10 is an anti-inflammatory cytokine and a major regulator of intestinal homeostasis (Hanyang et al., 2017; Daskalaki et al., 2021). DSS is associated with the downregulation of intestinal *il10* in mice (Silvestri et al., 2020), as we found in this study, though with no statistical differences to the CTRL. However, curcumin, was able to improve the expression of *il10*, though without statistical differences to the CTRL-D.

Inflammation and oxidative stress are pathological processes known to be related (Hewlings & Kalman, 2017). The inflammatory cells release a high number of oxygen and nitrogen reactive species (ROS and NOS) in the inflammation site, leading to oxidative stress (Hewlings & Kalman, 2017). On the other hand, oxidative stress triggers an intracellular signalling cascade that enhances the pro-inflammatory gene expression (Hewlings & Kalman, 2017). The release of ROS and NOS, and further intestinal inflammation, results in intestinal ischemia and necrosis, creating a vicious cycle (Burge et al., 2019). As a result, inflammation-related signalling pathways are upregulated, such as the NFkB pathway (Cao et al., 2015). At low concentrations, ROS are an indispensable defence mechanism to control microorganisms, however, inadequate removal of their intermediate compounds can lead to oxidative stress (Mise Yonar et al., 2017). The antioxidant nonenzymatic (total glutathione) and enzymatic (superoxide dismutase, catalase, glutathione peroxidase, glutathione s-transferase and glutathione reductase) systems are the first lines of defence against oxidative stress (Mise Yonar, 2017). Superoxide dismutase and glutathione peroxidase activities are crucial to stabilize cell membranes and to protect the intestinal tissue from the homeostasis instability caused by excessive production of ROS and NOS, but superoxide dismutase and glutathione peroxidase activities decreased in the intestine after the ingestion of DSS in mice (Xiang et al., 2021). Without this antioxidant capacity, the intestine is not able to repair the intestinal wall and alleviate the inflammation (Xiang et al., 2021).

In the present study, superoxide dismutase activity, as well as *sod* and *gpx* mRNA levels decreased in the intestine of seabream orally administered DSS, thus hampering the antioxidant capacity of the intestine to overcome the production of associated ROS and NOS, allowing a significant accumulation of free radicals in the intestine, and contributing to the lipid peroxidation (Cao et al., 2015). In fact, lipid peroxidation increased significantly in fish fed CTRL-D and BG-D, while curcumin was able to improve the intestinal response to lipid peroxidation induced by the DSS chemical and tended to increase *sod* and the *gpx* transcripts. Curcumin has a strong ability to stimulate antioxidant enzyme activities and to inhibit lipid peroxidation (Ali et al., 2006; Manju et al., 2009; Manju et al., 2012; Cao et al., 2015; Jiang et al., 2016; Mahmoud et al., 2017; Mise Yonar et al., 2017; Yonar, 2018; Giri et al., 2019; Yonar et al., 2019; Mohamed et al., 2020; Rajabiesterabadi et al., 2020; Alagawany et al., 2021; Xavier et al., 2021), reducing the levels of ROS and NOS, responsible for the breakdown of intestinal tight junctions, tissue injury and necrosis (Burge et al., 2019). The increase of the relative mRNA expression of *sod, cat, gpx, gr,* and *gst* after curcumin supplementation was found in previous studies (Cao et al., 2015 and Jiang et al., 2016). The antioxidant mechanisms of curcumin in the gene expression seems to be related to the activation of the nuclear factor erythroid 2-related factor (NRF2) signalling pathway, which plays a major role

in improving the gene expression of codifying genes for detoxifying, antioxidant, and anti-inflammatory proteins (Akdemir et al., 2017; Jiang et al., 2016; Alagawany et al., 2021).

The intestinal inflammation, necrosis, and oxidative stress impair the intestinal barrier and the microbiota community (dysbiosis), allowing the bacterial translocation and, ultimately, systemic inflammation (Eichele & Kharbanda, 2017; Burge et al., 2019). The haematological parameters are an important tool to establish fish physiological status and health, also to provide important information for diagnosis and prognosis of diseases (Fazio, 2019). Hemograms are related to erythropoiesis and anaemia (Fazio, 2019), while leucograms are indispensable to evaluate stress and diseases in fish (Fazio, 2019; Seibel et al., 2021). The presence of high numbers of circulating leukocytes is indicative of inflammation or infection (Fazio, 2019; Seibel et al., 2021). In the present study, haematocrit and red blood cells counts were increased after the dietary treatment with DSS, while haemoglobin was reduced. β-glucans and curcumin improved the haematocrit and haemoglobin content, indicating that these feed additives had a beneficial effect on the metabolism and availability of nutrients in the bloodstream (Ashry et al., 2021), contributing with more oxygen and nutrients to the cells at the inflammation site (Fazio, 2019; Seibel et al., 2021). The enhancement of the haematological parameters can be found after the dietary supplementation with curcumin in fish (Elgendy et al., 2016; Mahmoud et al., 2017; Yonar, 2018; Yonar et al., 2019; Moahmed et al., 2020; Ashry et al., 2021). The improvement of haemoglobin production was also found in broilers by Sugiharto et al. (2011), after the ingestion of curcumin, though the concentration of erythrocytes and haematocrit were not affected by this feed additive. On the other hand, laminarin was able to reduce the red blood cells counts and haemoglobin content in fish infected with *Aeromonas hydrophila* (El-Feki et al., 2000). As DSS inflammation impairs the intestinal barrier and may allow bacterial translocation, the increase of white blood cells counts in plasma is essential to control the bacterial population and to prevent a systemic infection (Ashry et al., 2021). In this study, the increase of circulating thrombocytes in fish fed CTRL-D and BG-D suggest a certain degree of thrombocytes proliferation and mobilization due to DSS treatment, a fact that seems to be counteracted by dietary curcumin. Moreover, BG-D dietary treatment was able to increase circulating lymphocytes and further suggest certain immunomodulatory properties of BG in response to DSS treatment that deserved further attention.

As described by Nordvi et al., 2023, there is a lack of studies related to intestinal damage and inflammation in fish models, especially related with low and moderate reversible inflammation. In this study, DSS produced mild effects on gilthead seabream health parameters, and, therefore, it can be suggested as a good model to study the intestinal inflammatory process in fish. However, other studies should be conducted to stablish DSS dose and effect, to define DSS as a robust model to study fish intestinal inflammation. Also,

this study showed that the conjugation of these two feed additives seems to be promising, reverting mild signs of inflammation and strengthening the defence mechanisms of gilthead seabream towards pathogens, improving the response to intestinal inflammation, and preventing the transition of bacteria from the intestine into the bloodstream, however, further studies are needed to stablish concentration and to fully understand the mechanisms involved in the process.

5. Conclusions

In conclusion, the dietary administration of DSS affected health parameters of gilthead seabream, such as the haematological profile, the intestinal oxidative stress biomarkers, and the intestinal gene expression profile, indicating that DSS can be a useful model to study intestinal inflammation in fish. Also, the adverse effects induced by DSS seemed to be partially alleviated by dietary supplementation with curcumin, that improved the haematological profile and helped against oxidative stress. However, further studies are needed to fully understand the correct amount of DSS as a chemical inducer of intestinal inflammation, as well as the feed additives concentration necessary to prevent intestinal inflammation, and the associated mechanisms involved in the process.

6. Acknowledgments

This work was partially supported by Projects FICA (reference ALG-01-0247-FEDER-047175), UIDB/04423/2020 and UIDP/04423/2020, financed by Portugal and the European Union through FEDER, COMPETE 2020, and CRESC Algarve 2020, in the framework of PORTUGAL2020, and through the COMPETE and Operational Human Potential Programs and national funds through Fundação para a Ciência e a Tecnologia (FCT, Portugal). This study was also partially funded by project ATLANTIDA (ref. NORTE-01-0145-FEDER-000040), supported by the North Portugal Regional Operational Program (NORTE2020), under the PORTUGAL2020 Partnership Agreement and through the European Regional Development Fund (ERDF). C. Teixeira and B. Costas were supported by FCT, Portugal (PD/BDE/135541/2018 and 2020.00290.CEECIND, respectively). C. Teixeira was also supported by SPAROS through grant PD/BDE/135541/2018. Pereira G.V. was supported by FCT recruitment call of CEECIST/00168/2018.

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Chapter V

Effect of krill meal and a triglyceride-rich krill oil on the immunity and intestinal microbiota of the Pacific white shrimp (*Penaeus vannamei*) fed a high-soybean meal diet

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Abstract

There is a generalised trend towards a reduction of fish meal (FM) and fish oil (FO) use in shrimp feeds. This study evaluated the effect of FM and FO replacement by krill meal (KM) and a triglyceride-rich krill oil (KO) in a high soybean meal-based diet on immunity, gut histology, and intestinal microbiota of Pacific white shrimp (*Penaeus vannamei*). Two diets were formulated with 50% of soybean meal. The control diet (CTRL) presented 5% of FM and 1% of FO whereas 50% of FM and FO were replaced by KM and KO in the krill diet (KRILL). Pacific white shrimp juveniles weighing 1.2 ± 0.5 g were randomly distributed among 10 glass tanks of 90 L of water capacity (45 shrimp each tank), in a saltwater recirculating system. Shrimps were fed the experimental diets (5 replicate tanks per diet) for 30 days. At the end of the trial, 8 shrimps were randomly selected from each tank, weighed, and sampled to evaluate plasma immune parameters, gut histology, intestinal gene expression, and intestinal microbiota. Besides a similar zootechnical performance, shrimps fed with the KRILL diet, showed no major alterations in the immune system and the intestinal morphology criteria. On the other hand, the presence of beneficial bacteria was verified in the shrimp fed with the KRILL diet. The results obtained in this study indicate that KM and KO can replace FM and FO in extreme plant-based diets for shrimps, with beneficial effects in the microbiota of shrimps.

1. Introduction

Protein consumption presented an exponential growth in the last years (FAOSTAT, 2018). Pacific white shrimp (*Penaeus vannamei*) farming, due to its importance as a protein source for human consumption, fast growth rates, short culture periods and high export value, has expanded rapidly across the world (Ayisi et al., 2017; FAO, 2020). Nutrition is extremely important in shrimp aquaculture, representing up to 50% of the production costs (Ayisi et al., 2017), and protein represents the most expensive nutrient in commercial diets for shrimps (Tacon & Metian, 2008; FAO, 2011; Ayisi et al., 2017). High digestible protein and an accurate lipid content are indispensable to maintain the correct balance between essential amino acids and lipid content, and to achieve maximum growth, health, welfare, and reproduction in shrimps (Nunes et al., 2010; Nunes et al., 2011). Practical feeds for Pacific white shrimp, typically contain 15-20% fishmeal (FM) and low levels of fish oil (FO) (0-2%). Given its large production volumes, the shrimp industry is one of the dominant users of FM within the aquaculture sector (Tacon & Metian, 2008; Kuresh & Davis, 2000; Ayisi et al., 2017; Ambasankar et al., 2022).

Since the global demand of FM and FO for aquafeeds is increasing, the availability is conditioned by fisheries (Tacon & Metian, 2008; FAO, 2011), and aquaculture production continues to grow (4% in 2019), prices of FM and FO are escalating (Tacon & Metian, 2008; FAO, 2011; Alltech, 2020). Consequently, the cost of commercial diets is increasing, and the high-intensive farming production is at risk (Tacon & Metian, 2008; FAO, 2011; Ayisi et al., 2017). Therefore, future FM inclusion level in shrimp feed is expected to further decline.

Soybean meal (SBM) is already a major ingredient in shrimp feeds (20-35% of formula). However, formulations with high SBM levels it is common to include low levels of other marine protein meals and hydrolysates to mitigate potential issues on palatability, amino acid imbalance, poor digestibility, and immune robustness (Yun et al., 2017). Krill in particular is a promising marine resource to be used in aquafeeds, due to its amino acid and fatty acid content, palatability, and pigment content (FAO, 2002; Burri & Nunes, 2016). KM, KO and krill hydrolysate are ingredients that can be used as feed stimulants in shrimp aquaculture to enhance feed palatability, increase feed intake, promote growth, and increase muscle quality, even in stressful conditions such as challenging plant-based diets or changes in the environmental conditions (Chien & Jeng, 1992; Córdova-Murueta & García-Carreño, 2002; Smith et al. 2005; Williams et al. 2005; Nunes et al., 2010; Nunes et al., 2011; Suresh et al., 2011; Sabry-Neto, et al., 2013; Derby et al., 2016; Sabry-Neto et al., 2017; Castro et al., 2018; Nunes et al., 2019; Nunes et al., 2020; Rufino et al., 2020; Soares et al., 2021; Ambasankar et al., 2022; Liang et al., 2022). In several fish species (*Salmo salar*, *Hippoglossus hippoglossus*, *Gadus morhua*, *Pagrus major*, *Paralichthys olivaceus*, *Larimichthys crocea*)

and shrimp (*Penaeus vannamei*) KM and KO has also been associated with improved intestinal development, enhanced innate immunity, and strengthened disease resistance (Olsen et al., 2006; Suontama et al., 2007; Hansen et al., 2010; Wei et al., 2019; Tharaka et al., 2020; Ambasankar et al., 2022; Liang et al., 2022).

To our best knowledge, there are still few studies focusing on the use of KM or KO to stimulate immunity and intestinal health in the Pacific white shrimp. Therefore, the present work aimed to contribute to this endeavour and investigate the role of KM and KO as immune enhancers and modulators of intestinal microbiota while replacing FM and FO in high-SBM diets for shrimp.

2. Material and methods

2.1. Experimental design and diets

European and Portuguese guidelines on the protection of animals used for scientific purposes, such as, FELASA category B and C recommendations, “Directive 2010/63/UE” and “Decreto-Lei n.º 113/2013 de 7 de Agosto” were applied to this trial. The experimental trial was executed at University of Trás-os-Montes e Alto Douro (UTAD) facilities, Vila Real, Portugal.

Juvenile shrimps originating from Blue Genetics (La Paz, Mexico), weighing 1.2 ± 0.5 g, were randomly distributed among 10 glass tanks of 90 L of water capacity (45 shrimp each tank) in a saltwater recirculating system. A photoperiod of 12 h light and 12 h dark was applied. Water dissolved oxygen was 6.8 ± 0.3 (mg L^{-1}), temperature 27.7 ± 1.0°C, pH 7.2 ± 0.1, and salinity 20 ppm. All animals were acclimatized to the experimental conditions for 10 days and fed with a commercial-type diet that fulfilled the known nutritional requirements for *P. vannamei*. The shrimps were fed four times a day, at 6% of body weight. After the acclimatization period, shrimps were had-fed *ad libitum* for 30 days with the experimental diets, in 5 replicate tanks for each diet (Table 1).

The trial comprised two experimental diets. A control diet (CTRL) with low levels of fish meal (5%) and fish oil (1%) and high levels of solvent-extracted soybean meal (50%); and a second diet in which 50% of fishmeal and fish oil were replaced by krill meal and krill oil, respectively, while maintain all other ingredients almost constant. One element worth mentioning is the fact that the krill oil used (Qrill AstaOmega oil, Aker Biomarine AS) is a product with the fatty acids mainly in the triglyceride form, rather than the most typically high-phospholipid krill oil. Both diets were supplemented with an inorganic phosphorus source and selected crystalline amino acids to avoid any nutritional deficiencies. Diets were

isonitrogenous (crude protein 42% dry matter), isolipidic (8.6-8.9% dry matter) and isoenergetic (20.3-20.6 gross energy).

Table 1: Ingredient and chemical composition of the experimental diets used in this study.

Ingredients (% feed) *	CTRL[1]	KRILL[2]
Fishmeal	5.00	2.50
Krill meal		2.50
Wheat gluten	2.50	2.50
Soybean meal	50.00	50.00
Rapeseed meal	5.00	5.00
Wheat meal	20.50	20.70
Rice bran full fat	8.00	8.00
Fish oil	1.00	0.50
Krill oil		0.50
Soybean oil	3.50	3.30
Rapeseed lecithin	1.00	1.00
Vitamin and mineral premix	1.00	1.00
Monoammonium phosphate	1.40	1.40
L-Lysine	0.50	0.50
L-Threonine	0.10	0.10
DL-Methionine	0.50	0.50
Proximate Analyses (% dry weight)		
Crude protein	41.74	41.78
Crude fat	8.58	8.92
Ash	7.41	6.47
Gross energy	20.34	20.61
Phosphorus	1.45	1.04

[1]**CTRL** (control diet), [2]**KRILL** (krill diet). *** Fishmeal:** CONRESA 60 - 65% CP, 10% CF, Conserveros Reunidos S.A., Spain; **Krill meal: QRILL Aqua -** 52% CP, 22% CF, Aker Biomarine, Norway; **Wheat gluten:** 81% CP, 2.1% CF, Roquette, France; **Soybean meal:** Solvent extracted soybean meal - 43% CP, 2.7% CF, CARGILL, Spain; **Rapeseed meal:** Defatted rapeseed meal - 32.7% CP, 4.1% CF, Ribeiro & Sousa Lda, Portugal; **Wheat meal:** 10.2% CP; 1.2% CF, MOLISUR, Spain; **Rice bran full fat:** 13.3% CP; 16.3% CF, Ribeiro e Sousa Lda, Portugal; **Fish oil:** Sopropêche, France; **Krill oil:** QRILL AstaOmega Oil, Aker Biomarine, Norway; **Soybean oil:** J.C. Coimbra, Portugal; **Rapeseed lecithin:** LECICO GmbH, Germany; **Vitamin and mineral premix:** PREMIX Lda, Portugal - Vitamins (IU or mg kg $^{-1}$ diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 500 mg; inositol, 500 mg; biotin, 3 mg; calcium pantothenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg kg $^{-1}$ diet): copper sulfate, 9 mg; ferric sulfate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulfate, 7.5 mg; sodium chloride, 400 mg; excipient wheat middling's; **Monoammonium phosphate:** Windmill Aquaphos - 26% P, ALIPHOS ROTTERDAM B.V, The Netherlands; **L-Lysine:** L-Lysine HCl 99% - Ajinomoto Eurolysine SAS, France; **L-Threonine:** ThreAMINO 98.5%, Evonik Nutrition & Care GmbH, Germany; **DL-Methionine:** Rhodimet NP99, ADISSEO, France.

Diets were produced by Sparos Lda (Olhão, Portugal). All powder ingredients were mixed accordingly to the target formulation in a double-helix mixer (model 500L, TGC Extrusion, France) and ground (below 400 µm) in a micropulverizer hammer mill (model SH1, Hosokawa-Alpine, Germany). Diets (pellet size: 1 mm) were manufactured with a twin-screw extruder

(model BC45, Clextral, France) with a screw diameter of 55.5 mm. Extrusion conditions: feeder rate (32-34 kg h^{-1}), screw speed (60-64 rpm), water addition (455 ml/min), temperature barrel 1 (32-34°C), temperature barrel 3 (97-102°C). Extruded pellets were dried in a vibrating fluid bed dryer (model DR100, TGC Extrusion, France). After cooling, oils were added by top coating (model PG-10VCLAB, Dinnissen, The Netherlands).

2.2. Sample collection

At the end of the trial, 8 shrimps were randomly selected from each tank and weighed. Two shrimps were used for haemolymph collection (n = 10 per diet), and the other 6 were euthanized in ice bath and sampled for tissue collection. From these 6 animals, 2 were used to collect intestine for gene expression (n = 10 per diet), other 2 to collect intestine for histology (n = 10 per diet), and the last 2 shrimps were used to collect intestine for microbiota analysis (n = 10 per diet). The animals were fasted for 12 hours prior to the histology and gene expression intestinal collection, and afterwards, the shrimps were fed 30 minutes prior to the intestinal microbiota collection.

Haemolymph samples were taken using a syringe (1 mL) pre-chilled with 100 µL of an anticoagulant solution (27 mM trisodium citrate, 385 mM sodium chloride, 115 mM glucose, pH 7.5), and the final volume was adjusted to a final dilution of 1:1 (v:v). The intersegmental membrane between the cephalothorax and the first abdominal segment was used to collect the haemolymph for the assessment of plasma innate immune parameters. The haemolymph was transferred to a 1.5 mL microtube, centrifuged at 2500 rpm for 10 min at 4°C. Then the plasma was placed in a new microtube, and the haemocytes were discarded. The plasma was snap-frozen in liquid nitrogen and stored at -80°C.

For both the intestinal gene expression and the microbiota analyses, the cephalothorax was pulled out, exposing the hepatopancreas and the intestine. With a sterilized scissor, the exoskeleton and the muscle were cut, and the total intestine was removed and stored in RNAlater (24h at 4°C and then -20°C; Sigma) for intestinal gene expression analysis, or in 96% molecular ethanol for microbiota analysis, until assayed. The same procedure was used for histology tissue collection and intestinal samples were fixated in a 4% buffered formalin solution (Sigma) for 48 h.

2.3. Plasma humoral immunological parameters

The colorimetric Pierce™ BCA Protein Assay Kit (Thermo Scientific™) was used to determine the total plasma protein concentration, according to manufacturer recommendations, with adaptations for 96-well microplates. Briefly, 10 µL of plasma were

diluted in 990 µL of distilled water (1:100 dilution) in a microtube. Twenty-five µL of this dilution was then transferred to each 96-well microplate. Total protein (mg mL^{-1}) was calculated based on the standard curve made with bovine serum albumin.

Phenoloxidase activity was measured according to Ji et al. (2009) with some modifications. Briefly, 25 µl of plasma were incubate with 100 µl trypsin (1 mg ml^{-1}) for 30 min at room temperature. Then, 100 µl L-DOPA (L-3,4-dihydroxyphenylalanine, Sigma) (3 mg ml^{-1}) were added and the absorbance was measured, each minute, during 5 minutes at 490 nm. Results were expressed as units of phenoloxidase per mL of plasma.

The anti-protease activity of plasma was established following the method of Machado et al. (2015), with some modifications. Briefly, 20 µL of plasma were incubated with 10 µL of a trypsin solution (5 mg mL^{-1} in $NaHCO_3$, pH 8.3, Sigma) for 10 min at room temperature. Phosphate buffer (NaH_2PO_4, 13.9 mg mL^{-1}, pH 7.0, Sigma) was used instead of plasma as a reference sample. For blank, phosphate buffer was used instead of plasma and trypsin solution. Then, 90 mL of phosphate buffer and 125 µL of azocasein (20 mg mL^{-1} in $NaHCO_3$, 5 mg mL^{-1}, pH 8.3, Sigma) were added and incubated for 1 h, at room temperature, in dark. After the incubation, 250 mL of trichloroacetic acid (100 mg mL^{-1}, Sigma) were added to each microtube and incubated for 30 min at 22°C. The mixture was then centrifuged at 10000 × g, for 5 min, at room temperature. Afterwards, 100 mL of the supernatant was transferred to a 96 well-plate that previously contained 100 mL of NaOH (40 mg mL^{-1}, Sigma) each well. The optical density (OD) was read at 450 nm and the antiprotease activity inhibition (%) was calculated in comparation to the reference sample.

A turbidimetric assay was used to evaluate lysozyme activity, as described by Machado et al. (2015), with some modifications. Briefly, a standard curve was developed using a serial dilution of a lyophilized hen egg white lysozyme (Sigma) in sodium phosphate buffer (Na_2HPO_4, 7.098 mg mL^{-1}, pH 6.2, Sigma). Twenty µL of the standard curve and plasma were incubated with 150 µL of a *Micrococcus lysodeikticus* solution (0.25 mg mL^{-1} in Na_2HPO_4, 7.098 mg mL^{-1}, pH 6.2, Sigma) at room temperature. Sodium phosphate buffer was used instead of plasma as blank. The OD was read at 450 nm at 0 and 5 min, and the activity of lysozyme (µg mL^{-1}) in plasma was calculated using the standard curve.

The plasma bactericidal activity was performed as described by Machado et al. (2015) and Naghili et al. (2013) with some modifications. Briefly, *Vibrio harveyi* (IA20.2, kindly provided by Prof. Alicia Toranzo) was cultured in Tryptic Soy Agar (TSA), overnight, at 25°C. The bacterial colonies were then resuspended in Tryptic Soy Broth (TSB) and adjusted to OD read of ≈ 0.300 at 600 nm. This suspension was then serially diluted with TSB and cultured on TSA, to establish colony-forming units (CFUs), using the drop plate technique with 10 µL of bacterial dilution. The bacterial suspensions correspond to 2 x 10^9 CFUs ml^{-1}. Plasma bactericidal activity was then determined using 40 µL of plasma suspended in 20 µL of *Vibrio harveyi*

solution in a U-shaped 96-well plate and incubated for 2.5 hours, at 25°C, with shaking. TSB was used instead of plasma as reference sample and 60 µL were added to another well as blank. To each well, 25 µL of 3-(4,5 dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, 1 mg mL^{-1}; Sigma) were added and incubated for 10 min, at 25°C, with shaking, to allow formazan formation. Plates were then centrifuged at 2000 × g for 10 min and the supernatant was discarded. The precipitate was resuspended in 200 mL of dimethyl sulfoxide (DMSO, Sigma) and the OD of the dissolved formazan was measured at 560 nm. Bacterial survival was calculated comparing the samples and the positive control (maximum survival). Total bactericidal activity was expressed as the percentage of killed bacteria, calculated from the difference between the samples and the positive control (100% living bacteria).

All the assays were done on a Synergy™ HT microplate reader (Bio-Tek®), in duplicates.

2.4. Intestinal histology

The intestinal sections were processed according to Guardiola et al. (2013), with some modifications. Briefly, the fixated intestine was transferred to growing concentrations of ethanol, for dehydration, and then, the sample was placed in a xylene solution for transparency. Afterwards the sample was embedded in paraffin and sectioned transversally in to 2 µm sections. The intestinal sections were mounted and stained with hematoxylin-eosin (H-E). Villi height, width and area were measured using a light microscope (Nikon Eclipse E200) equipped with a camera (Sony, E3CMOS06300KPA) and appropriate software (ToupView 3.7 for digital camera).

2.5. Intestinal gene expression

For RNA extractions, the shrimp's intestine was placed in a 2 mL tube containing 0.5 mL of Trizol (Nzol Reagent, NZYTech, Portugal) and two Zirconium Oxide-coated ceramic grinding sphere (2 mm diameter) and homogenised in a Precellys 24 tissues homogenizer (Bertin Ins., France) by 2 cycles of 6000 x g for 20 seconds. After that, 150 µl of chloroform was added to the homogenate, gently vortexed and centrifuged for 15 min at 12000 x g and 4°C. Around 300 µl of the aqueous phase were transferred to a new 1.5 ml tube with 300 µl of 70% ethanol and gently homogenised. After this step, the RNA extraction was done with a NZY Total RNA Isolation Kit (NZYTech, Lisbon, Portugal) following the manufacturer's specifications. Spectrophotometry and the 260:280 and 260:230 ratios were used to assess the quantity and purity of the RNA extracted in a DeNovix DS 11 FX Instrument (DeNovix, US). The integrity of the RNA obtained was verified through 1.5% agarose gel electrophoresis. NZY First-Strand cDNA Synthesis Kit (NZYTech, Lisbon, Portugal) was used to synthesize

first-strand cDNA, following the manufacturer's specifications. The NCBI Primer Blast Tool (Ye et al., 2012) were used to design the oligonucleotide primers used in this study (Table 2). As described by Machado et al. (2018), efficiencies were calculated in a serial five-fold dilutions of cDNA, using the slope of the regression line of the cycle thresholds (Ct) versus the relative concentration of cDNA. The amplification of primer dimers was also verified through melting curve analysis. iQ5 Real Time PCR detection System (Bio-Rad) was used for quantitative PCR assays. Four point four microliters of cDNA were mixed with 5 µl of iQ SYBR green 2x Supermix (Bio-Rad) and 0.3 µl (10 mM) of each specific primer in a final volume of 10 µL. Initially, one cycle of 95°C for 10 min was performed, followed by one cycle of 95°C for 15 sec and one cycle of 60°C for 1 min. After that, 40 cycles of 95°C for 15 sec were followed by one cycle of 95°C for 1 min and one cycle of 60°C for 30 sec. Then, a melting curve from 60°C to 95°C, with increments of 0.5°C for each 0.5 sec was performed, followed by a cycle of 95°C for 15 sec in a CFX384 Touch™ Real-Time PCR Detection System (BioRad)**.** Technical duplicates were carried out for all reactions. Beta-actin (*β-actin*) expression was used as reference gene to normalize the expression of the target genes.

Table 2: Oligonucleotide primers used in this study for Real-time PCR analysis of gene expression.

Target Gene name	Symbol	Accession nr.	Forward primer (5´-3´)	Reverse primer (5´-3´)	PL[1]	E[2]
Beta-actin	*bactin*	AF300705	GCCCTGTTCCAGCCCTCATT	ACGGATGTCCACGTCGCACT	93	2.06
Penaeidin-3a-like	*pen3*	XM_027360479.1	ATACCCAGGCCACCACCCTT	TGACAGCAACGCCCTAACC	137	2.22
Anti-lipopolysaccharide factor	*alf*	XM_027372864.1	TTACTTCAATGGCAGGATGTGG	GTCCTCCGTGATGAGATTACTCTG	142	1.98
Lysozyme C-like	*lzc*	XM_027352840.1	CGGGAAAGGCTATTCTGCCT	CCAGCACTCTGCCATGTACT	82	2.24
NFKB immune deficiency	*imd*	FJ592176.1	TATACATCCTGCCGTTGCCG	GGATAACGGGGCCAATGTGA	169	2.04
NFKB relish	*relish*	XM_027357251.1	CCAGGTCATGGTGAGAGGTG	TGCTCCATCGTGGTGAACAG	157	1.99
1,3-beta-D-glucan-binding protein precursor	*bgbp*	AY249858.1	GGACTGGTGCCCGAAAGAAA	TCTGCCATGGGGAGTTGTTAG	153	2.11
TAR RNA-binding protein 1	*trbp1*	HQ541157.1	CAAGAGTTGTGTATGCGCAG	AGCACCACCCTGGATGATG	195	2.03
Phenoloxidase 3-like	*po3*	XM_027379995.1	CAATGACCAGCAGCGTCTTC	CACGGAAGGAGGCGTATCAT	118	2.17
CD63 Ag/tetraspanin	*tetra*	XM_027356349.1	TCCTCTCGCACTGGTGTCTA	GCAGCAAGCATCAGGAACA	135	2.00
Thioredoxin-2-like	*trx2*	XM_027377405.1	TTCCTGAAGGTGGATGTGGA	AGTTGGCACCAGACAAGCTG	121	2.08
Metallothionein-1-like	*mt1*	XM_027367320.1	CTGATCCATGCTGTAACGAG	CATCTTGTTGCACACTCCTC	145	2.11
Inhibitor of apoptosis protein	*iap*	GQ293142.1	CAACACCTGCCTCAGGACAA	CTTCCATTGCCTCCTCGTCT	111	1.91
Heat shock cognate 70 kDa protein	*hsp70*	XM_027369405.1	CAACGATTCTCAGCGTCAGG	ACCTTCTTGTCGAGGCCGTA	117	2.09
Trypsin-1-like	*tryp*	XM_027367621.1	CGGAGAGCTGCCTTACCAG	TCGGGGTTGTTCATGTCCTC	141	2.17

[1] Product length or amplicon (nt)

[2] Efficiency of PCR reaction

2.6. Intestinal microbiota analysis

DNA extraction from shrimp intestinal samples was performed according to Serra et al. (2021), with some modifications. Briefly, intestinal samples from 2 shrimps per tank were pooled (n = 5 per diet), suspended and homogenized 3 times. The following incubations were performed as described by Serra et al. (2021), however the centrifugation was carried out at 13000 rpm for 10 min, and only 10 mg ml^{-1} of lysozyme (Sigma) were used. Then, the DNA pellet was washed twice with 500 µl of ice-cold 70% ethanol by centrifugation (13000 rpm, 10 min) and dried at 37°C for 10 min. The DNA was resuspended in 50 µl ultrapure water for 30 min at 37°C and stored at -20°C. An additional purification step was performed using the OneStep™ PCR Inhibitor Removal Kit (Zymo Research, USA.), following manufacturer recommendations.

The taxonomic diversity of the gut microbiota was assessed by Next-Generation Sequencing (NGS) technology. Five pools of each diet (2 shrimps per tank, 5 tanks per diet) were sequenced using the Illumina MiSeq platform (Macrogen Inc., Seoul, Rep. of Korea), targeting the V3-V4 hypervariable region of the 16S rRNA gene, to obtain a sequence informative length of 300 bp. Using the Flash program, the paired-end reads were merged to produce longer reads (Magoc & Salzberg, 2011). The CD-HIT program was used to do the pre-processing (e.g. removal of low-quality reads) and clustering (Li & Godzik, 2006). The filtered sequences were clustered at 100% identity into operational taxonomic units (OTUs) identifying the chimeric reads, and after the removal of the noise sequences (small size), the remaining representative reads from non-chimeric clusters were clustered into OTUs at a 97% ID to species level cut-of. Singletons were removed from the analysis. Quantitative Insights Into Microbial Ecology (QIIME 2) software (Caporaso et al., 2010) and SILVA138 16S reference database (Quast et al., 2013) were used for taxonomy assignment and diversity statistics. The metagenomic profiles were evaluated using the STAMP v2.1.3 software (Beikolab) (Parks et al., 2014) and taxonomic comparations were assessed employing Venny 2.1 software (BioinfoGP) (Oliveros, 2007-2015).

2.7. Statistical analysis

Data were expressed as means and standard deviation and analysed for normality and homogeneity of variance. A parametric T-test was conducted to confirm whether the two diets were statistically different from each other. When necessary, results were transformed before being treated statistically. The statistical analysis was performed using IBM SPSS Statistics

26 and STAMP v2.1.3 software (Beikolab) (Parks et al., 2014). The level of significance used was $p < 0.05$ for all statistical tests.

3. Results

After 30 days of experimental feeding, survival and zootechnical performance criteria (weight gain and feed conversion) were equivalent among the two dietary treatments.

3.1. Plasma humoral immunological parameters

Humoral immunological parameters in plasma are showed in Figure 2. Lower levels of total protein, as well as a significant decrease of phenoloxidase activity were found in the KRILL group compared to the CTRL. On the other hand, the antiprotease, lysozyme and bactericidal activities were slightly higher in the shrimps fed with the KRILL diet, though without statistical differences.

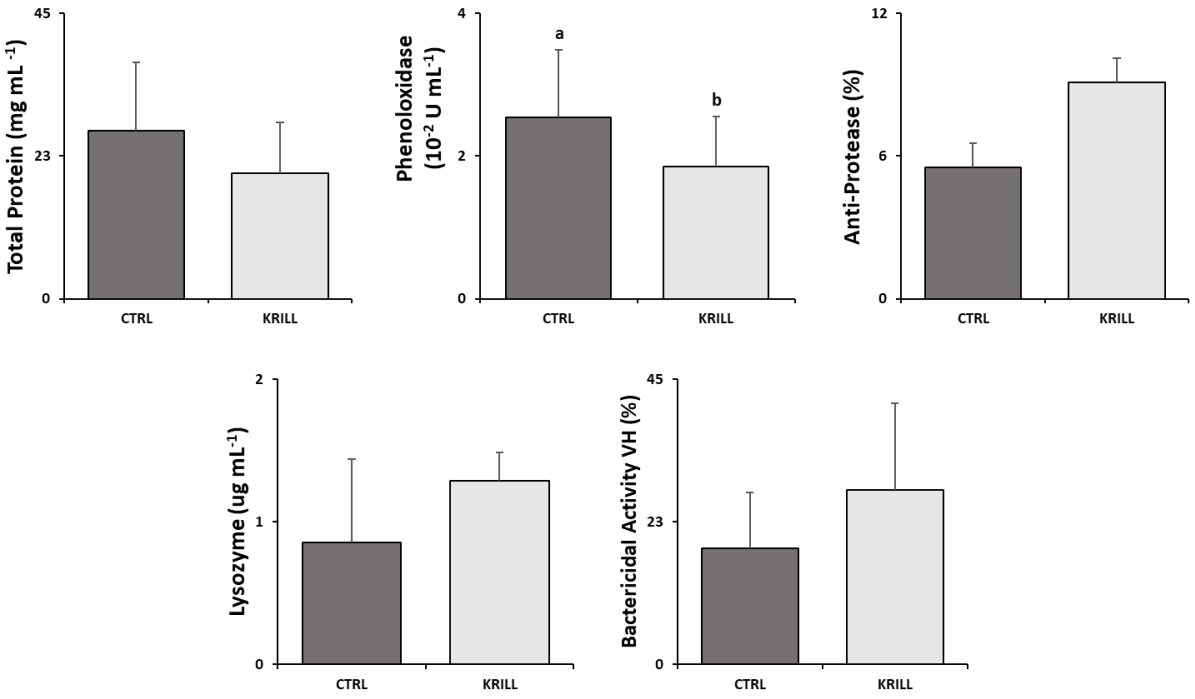

Figure 2. Humoral immunological parameters in plasma of Pacific white shrimp fed the experimental diets, **CTRL** (control diet) and **KRILL** (krill diet). Different letters mean significant differences among the dietary treatments ($p < 0.05$).

3.2. Intestinal histology

The histological outline of the intestine did not show any statistical differences between the dietary treatments, although the KRILL group presented a tendency for higher values of villus width (Figure 3).

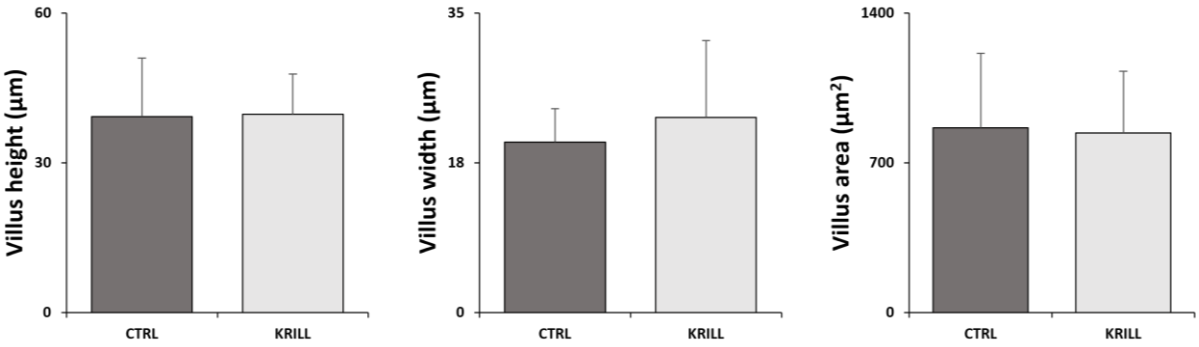

Figure 3. Intestinal histology of *P. vannamei* fed the experimental diets, **CTRL** (control diet) and **KRILL** (krill diet).

3.3. Intestinal gene expression

Table 3 describes the fold change of the gene expression profile of the intestine of Pacific white shrimp fed the CTRL and KRILL dietary treatments. There were no statistical differences between both dietary treatments in the target genes analysed. The antimicrobial peptide gene *alf*, the redox system genes *trx2* and *mt1*, and the cellular apoptosis gene *iap* were the most expressed genes in the shrimp intestine.

Table 3. Fold change of the gene expression profile in the intestine of Pacific White Shrimp fed the experimental diets **CTRL** (control diet) and **KRILL** (krill diet).

Target gene acronym	CTRL	KRILL
pen3	1.00 ± 0.90	0.76 ± 0.75
alf	1.00 ± 0.46	0.62 ± 0.56
lzc	1.00 ± 1.12	2.66 ± 1.93
imd	1.00 ± 0.79	1.41 ± 1.04
relish	1.00 ± 0.75	1.51 ± 1.21
bgbp	1.00 ± 1.15	2.00 ± 2.30
trbp1	1.00 ± 0.60	1.28 ± 0.52
po3	1.00 ± 1.32	1.95 ± 1.27
tetra	1.00 ± 1.56	0.60 ± 0.45
trx2	1.00 ± 0.29	1.20 ± 0.50
mt1	1.00 ± 1.04	0.43 ± 0.39
iap	1.00 ± 0.23	1.16 ± 0.39
hsp70	1.00 ± 0.43	1.53 ± 0.98
tryp	1.00 ± 1.06	0.65 ± 0.63

Values presented as means ± standard deviation.

Thought without statistical differences, the lytic enzyme gene *lzc* and the microbial recognition gene *bgbp*, presented at least two times more expression in the KRILL group than in the CTRL (Table 3).

3.4. Intestinal microbiota

Shrimp intestinal microbiota structure was assessed by 16S rRNA amplicon sequencing using the Illumina Miseq sequencing platform. At least 86500 read counts were provided per sample (Supplementary Table S1). After pre-processing, a total of 449906 high-quality reads were clustered into 210 OTUs at 97% identity threshold. Singletons were removed from the downstream analysis, resulting in 210 unique OTUs. The shrimp intestinal ecological parameters obtained from the NGS data (microbial richness, diversity, and evenness), are presented in Table 4 (raw data is in Supplementary Table S1). No statistical differences were found among the diets.

Table 4. Ecological parameters obtained from NGS analysis of the shrimp intestinal microbiota, when fed the experimental diets **CTRL** (control diet) and **KRILL** (krill diet).

Ecological parameters	CTRL	KRILL
Chao species richness [a]	87.37 ± 33.49	79.35 ± 30.01
Shannon's diversity index [b]	2.11 ± 0.68	2.25 ± 0.53
Simpson's Evenness index [c]	0.66 ± 0.17	0.70 ± 0.13

Values presented as means ± standard deviation. One-way ANOVA: * $p < 0.05$.

[a] **Chao species richness:** $S_{Chao1} = S_{obs} + n_1^2/2n_2$, where S_{obs} is nr of species, n_1 is singletons, and n_2 is doubletons

[b] **Shannon's diversity index:** $H' = -\sum(Pi(\ln Pi))$, whereas Pi is the nr of individuals of the ith species

[c] **Simpson's Evenness index:** $E = (1/\sum Pi2)/S$, where S is ty number of species

The total of sequences obtained were assigned into 12 phyla, 26 classes, 52 orders, 86 families, and 159 genera. Unclassified bacteria represented more than 2% of the total sequencing reads. The most abundant taxa, showing a mean proportion of 0.3% or higher in any dietary condition, are presented in Figure 4. *Proteobacteria* was the dominant phyla, accounting for more than 72% of the sequencing reads in CTRL and 77% in KRILL, followed by *Bacteroidetes* (around 20%). Other phyla, such as *Cyanobacteria*, *Actinobacteria*, *Firmicutes* and *Verrucomicrobia*, represented less than 1% of the sequencing reads, in both diets. *Gammaproteobacteria* and *Flavobacteria* represented more than 70% and 20%, respectively, of the classes found, with *Vibrionales* (more than 60%) and *Flavobacteriales* (around 20%) being the most represented orders, in both diets (Figure 4). *Vibrionaceae* (more than 60%), *Weeksellaceae* (around 20%) and *Shewanellaceae* (around 10%) were the most significant families observed in both diets, and the predominant genera were *Vibrio* (more than 55%), *Spongiimonas* (around 20%) and *Shewanella* (around 10%), also in both diets (Figure 4).

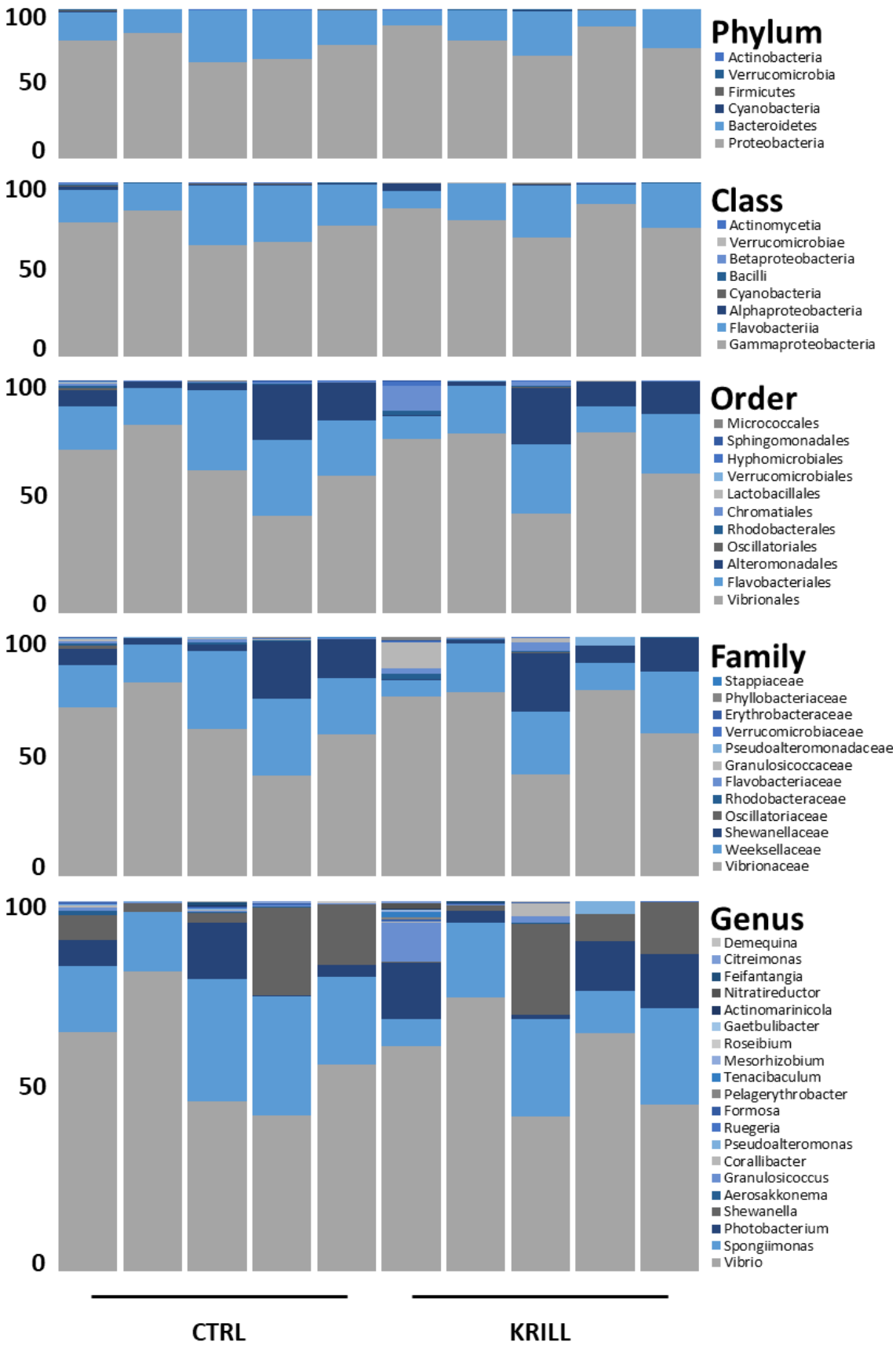

Figure 4. Relative bacterial abundance (y-axis) at Phylum, Class, Order, Family and Genus Taxonomic levels (from top to bottom), found in intestinal samples of Pacific White Shrimp fed the experimental diets **CTRL** (control diet) and **KRILL** (krill diet). Presented are taxa with a mean proportion ≥0.3% in any experimental feeding condition.

No significant differences were found between the dietary treatments in the taxonomic levels analysed (Phylum, Class, Order and Genus) (Figure 5), except for the Family taxa *Nocardiaceae*, which was more abundant in the KRILL than in the CTRL group (p = 0.039).

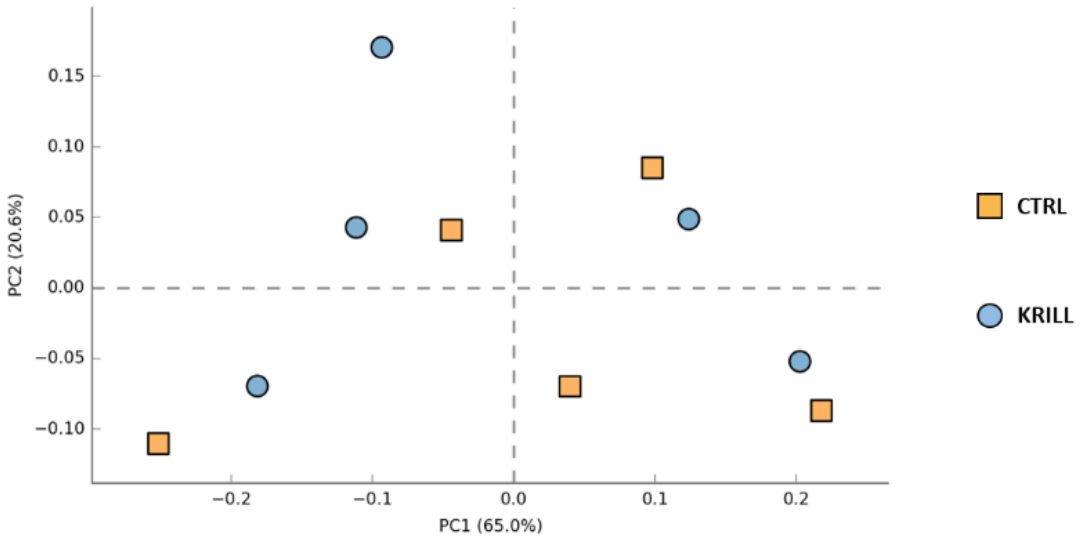

Figure 5. Principal component analysis (PCA) of the relative bacterial abundance at Genus taxonomic level found in intestinal samples of Pacific White Shrimp fed with the control diet (**CTRL** group) or the krill diet (**KRILL** group).

Regardless the diets provided, the great majority of the genera (92) were present in the two experimental groups, with 44 genera appearing exclusively in Pacific white shrimps fed the CTRL diet and 23 in the intestines of the ones fed the KRILL diet (Figure 6).

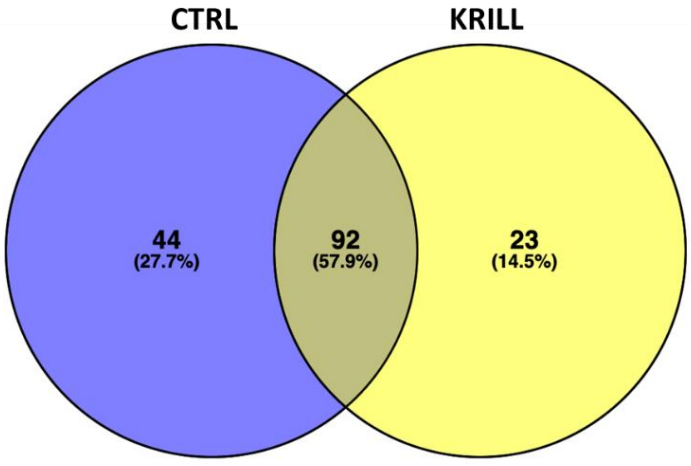

Figure 6. Venn diagram representation of shared and unique genera found in intestinal samples of Pacific White Shrimp across the experimental feeding groups **CTRL** (control diet) and **KRILL** (krill diet), using Venny (https://bioinfogp.cnb.csic.es/tools/venny_old/venny.php).

4. Discussion

Shrimp faster growth rates, short culture periods and high export value are rap-idly expanding shrimp aquaculture across the world (Ayisi et al., 2017; FAO, 2020). As a consequence, there is increased pressure on the reduction of FM and FO use in shrimp feeds. Soybean meal is the most common plant protein source used in aquafeeds to replace FM (FAO, 2011; Sookying & Soller, 2013; Derby et al., 2016; Sánchez-Muros et al., 2018), however it has some nutritional drawbacks (Nunes et al. 2011; Sookying & Soller, 2013; Burri & Nunes, 2016; Derby et al., 2016). Because shrimp species from the Penaeidae family are omnivores, and Pacific white shrimp is able to modulate its digestive enzymatic production according to the dietary components, challenging plant-based diets can be used in shrimp aquaculture (Córdova-Murueta & García-Carreño, 2002; Tzuc et al., 2014; Sabry-Neto et al., 2017). Nevertheless, Penaeids have some limitations in the ability to synthesize some essential fatty acids, and a minimum provision in the diet is mandatory (Sá et al., 2013). Krill meal and oil are high quality ingredients. Besides a well-balanced profile of amino acids and fatty acids in the form of phospholipids, they also contain astaxanthin, chitin, and soluble compounds such as trimethylamine oxide, free amino acids and nucleotides that may act as phagostimulants in shrimp (FAO, 2002; Burri & Nunes, 2016). Therefore, krill-based ingredients can be useful to improve the nutritional quality of a challenging plant-based diet and enhance the immune robustness of shrimp (FAO, 2002; Burri & Nunes, 2016). Previous research showed that Pacific white shrimp fed with plant-based diet with KM and whole squid meal inclusion (12 g kg^{-1}), for 30 days, presented an increase of final body weight, and were able to grow with only 5% of FM (Sá et al., 2013). According to Derby et al., 2016, 6% of KM was very effective on the attractiveness and palatability of plant-based diets for shrimp. One percent was the lower inclusion amount of KM in a plant-based feed to produce higher levels of feed intake. Nonetheless, 2% inclusion was necessary to induce growth, increased yield, and reduced feed conversion ratio (Sabry-Neto et al., 2017). According to Nunes et al., 2019, in a low FM diet supplemented with 3% of KM, for 74 days, krill was an effective feeding attractant, a palatability enhancer, and a growth promoter for juvenile Pacific white shrimp. In the present study, the 50% replacement of FM and FO by KM and KO in a challenging high-soybean meal diet, did not affect overall growth performance of *P. vannamei* juveniles. The short duration of the trial (30 days), the low dietary levels of KM (2.5%) and KO (0.5%) and the highly stringent formulation scenario (with 50% soybean meal), may justify the absence of a clear beneficial effect of the KRILL diet in terms of zootechnical performance.

Plasma humoral immunological parameters are valuable biological indicators of health and stress responses. In this study, total protein content of plasma was not affected by the replacement of half of the FM content by KM, indicating that the protein content remained

unaffected by this dietary substitution. There is a lack of studies focusing on the use of KM or KO to stimulate immunity in the Pacific white shrimp, however, despite the significant decrease of phenoloxidase in the KRILL group, the antiprotease, lysozyme and bactericidal activities tended to increase compared to the CTRL group. Phenoloxidase is stored inside the haemocytes in its inactive form (pro-phenoloxidase) and it is activated in the presence of antigen-derived molecules such as lipopolysaccharides (LPSs), β-1, 3-glucans, or peptidoglycan. In the activation process, this inactive enzyme is transported by exocytosis into the plasma (Cuéllar-Anjel, 2008) and activated by the prophenoloxidase activating enzyme (PPAE) (Cuéllar-Anjel, 2008; Jang et al., 2011). Therefore, the phenoloxidase activity allows to measure the immune response capacity of a shrimp, against the presence of pathogenic agents in the organism (Cuéllar-Anjel, 2008). Ambasankar et al., 2022 showed that the expression of *propo* and *ppae* genes were significantly higher in shrimp diets containing 12% of FM supplemented with 2%, 4% or 6% of KM, however, the expression of these genes was reduced in diets containing 6% of FM supplemented with 2%, 4% or 6% of KM. These results suggests that the inclusion of KM in high FM diets plays a considerable role in enhancing the immune status of shrimp, however, the reduction of FM in the diet compromises the immunological response of the pro-phenoloxidase cascade (Ambasankar et al., 2022), as verified in the present study.

As in the present study, in Atlantic salmon (*Salmo salar*), Olsen et al., 2006 and Hansen et al., 2010 did not observe any statistical differences in plasma immune parameters of fish fed with KM. Likewise, no statistical differences were observed by Suontama et al., 2007 in plasma total protein of Atlantic salmon (*Salmo salar*) and Atlantic halibut (*Hippoglossus hippoglossus*) fed diets with krill inclusion. However, Bui et al., 2014 found an improved total protein, antiprotease and lysozyme activities, in the red seabream (*Pagrus major*) fed with krill hydrolysate, which represented an improved immune response to a bacterial challenge with *Edwarsiella tarda*. Tharaka et al., 2020 described significant higher values of antiprotease and lysozyme activities, in the olive flounder (*Paralichthys olivaceus*) fed with low FM diets supplemented with KM. Although the direct effect of KM on the shrimp immune system is not well known, the nutritional profile of KM similar to FM, its richness in lipids, astaxanthin, and choline seems to have an important immune stimulatory effect (Tharaka et al., 2020) and has been associated with a robust response to stress in shrimps (Nunes et al., 2010; Castro et al., 2018; Nunes et al., 2020; Rufino et al., 2020).

Astaxanthin is the main carotenoid present in KM (Xie et al., 2019). Carotenoids have an important scavenging affinity to toxic oxygen radicals, especially from lipid peroxidation (Thompson et al., 1995), and thus, acts as a strong antioxidant and anti-inflammatory carotenoid (Chien and Jeng, 1992; Darachai et al., 1998; Pan et al., 2003). They have been associated with strong pigmentation, higher survival rate, improved growth, and strong

response to stress in shrimp (Chien and Jeng, 1992; Darachai et al., 1998; Pan et al., 2003). Antiproteases neutralize the proteases produced by the bacterial pathogens (Ellis, 1990b), and the antiprotease activity of plasma seems to be significantly improved by the dietary administration of astaxanthin (Thompson et al., 1995). Lysozyme is another important bactericidal agent present in plasma (Ellis, 1990a), that seems to benefit from the dietary supplementation of astaxanthin (Thompson et al., 1995). These findings are in line with those found in the present study, with clear tendencies to improve antibacterial proprieties and lytic enzymes in the shrimps fed with the KRILL diet (e.g. increased antiprotease, lysozyme and bactericidal activities).

As described previously, although KM stimulates the response of shrimp hepatopancreas R and B cells, increasing digestion and absorption (Burri et al., 2020), there were no statistical differences between the dietary treatments in terms of the intestinal histology from the present study. Hansen et al. (2010), Wei et al. (2019) and Henry et al. (2020), did not observe statistical differences in the intestinal histology of Atlantic salmon, large yellow croaker (*Larimichthys crocea*), and European seabass (*Dicentrarchus labrax*) fed with KM. Nevertheless, Tharaka et al. (2020) observed an increase of villi length in the olive flounder (*Paralichthys olivaceus*) fed with low FM diets supplemented with KM.

In the liver of large yellow croaker, the expression of genes related to the TOR signalling pathway, an important pathway in cell growth and proliferation, were not significantly affected by the replacement of FM by KM (Wei et al., 2019). In the present study, the gene expression of health-related genes was also not clearly affected by the dietary KM and KO inclusion in a challenging plant-based diet. More studies are needed to fully understand the effects of KM and KO in the intestinal and hepatopancreas morphology and health-related parameters in shrimps fed with vegetable ingredients and krill.

Despite the use of a challenging plant-based diet in the present study, the replacement of 50% FM and FO by KM and KO did not produce significant variations in the diversity and richness of the Pacific white shrimp intestinal microbiota. Higher levels of diversity in the gut microbiota community are thought to be beneficial for the host health, contributing to its stability, resistance, and resilience to environmental stressors (Naeem & Li, 1997; Lozupone et al., 2012; Valdes et al., 2018; Fan et al., 2019). On contrary, lower gut microbiota diversity is known to contribute to dysbiosis (microbial imbalance) and the development of diseases (Naeem & Li, 1997; Lozupone et al., 2012; Valdes et al., 2018; Fan et al., 2019). Fan et al., 2019 discovered that the diversity and dynamics of the shrimp gut microbiota composition were not remarkably affected by the dietary treatments used in that study but were highly connected to the shrimp weight.

Proteobacteria and Bacteroidetes were the predominant phyla in the present study, as found in previous studies (Rungrassamee et al., 2014; Rungrassamee et al., 2016; Fan et al.,

2019). *Vibrionales* order, *Vibrionaceae* family and *Vibrio* genera, were the most pronounced taxa found in the shrimp intestine microbial community in samples from this study, in agreement with previous works (Tzuc et al., 2014). Vibrio can be found in aquatic systems and in animal tissues as a part of the normal microbiota (de Souza Valente & Wan, 2021). Its presence can be highly affected by temperature, pH, salinity, and nutrients in the water, being normally seasonal and commonly detected during summer (de Souza Valente & Wan, 2021; Garibay-Valdez et al., 2020). Although regularly found in aquatic environment, some Vibrio species are known to be pathogenic, and shrimp aquaculture is very susceptible to Vibrio exposure (Tzuc et al., 2014; Chen et al, 2017; Fan et al., 2019).

Despite the different dietary regimes, a core microbiota, composed of approximately 60% of the genera found, was identified in the Pacific white shrimp intestine. All genera showing a mean proportion of 1% or higher in any dietary condition, considered the most abundant, belong to the core microbiota. These include genera commonly found in aquatic environments, some of which containing species described as highly problematic disease-causing pathogens in aquaculture (e.g. *Vibrio*, *Photobacterium* and *Tenacibaculum*) (Pridgeon, 2012). Most of shrimp pathogenic bacteria belongs to the Vibrio genera, however others such as Aeromonas, *Pseudomonas*, and *Flavobacterium* have also been implicated in shrimp diseases (Sivakamavalli et al., 2021). *Vibrio*, *Spongiimonas*, *Shewanella* and *Photobacterium* have been described as most abundant genera in previous bacterial profiles of shrimp intestine (Hou et al., 2020; Cheng et al., 2021). *Vibrio alginolyticus* and *V. xuii*, *Spongiimonas flava*, *Photobacterium damselae* and *Shewanella khirikhana* were the most commonly species found in this study. *Vibrio alginolyticus* can cause vibriosis infection in shrimp (Liu et al., 2004; Abdul Hannan et al., 2019), *Photobacterium damselae* is pathogenic for marine animals and humans (Rivas et al., 2013), and *Shewanella khirikhana* can be a shrimp pathogen (Prachumwat et al., 2020). Despite their presence, no signs of shrimp disease were seen during the trial, and no differences in the Vibrio population were verified between the dietary treatments. As described previously, Vibrio population is associated with shrimp's developmental stage and environment conditions and can be abundant in healthy shrimp intestines (Garibay-Valdez et al., 2020; de Souza Valente & Wan, 2021). Garibay-Valdez (Garibay-Valdez et al., 2020) argued that only an active metabolic intestinal Vibrio population is able to induce intestinal dysbiosis and disease, and the intestinal community structure, organized by different species probably regulates Vibrio activity, establishing a symbiotic relationship between the organism and the bacterial community structure of the intestinal microbiota.

The gut microbiota of Pacific white shrimps fed the KRILL diet was enriched for Nocardiaceae ($p = 0.039$) an aerobic and Gram-positive bacteria found worldwide (Rathish & Zito, 2021). This family included Nocardia and Rodococcus genera in this study. Nocardia is a filamentous bacillus know to be an opportunistic pathogen to human immunosuppressed

patients (Rathish & Zito, 2021). *Nocardia neocaledoniensis*, isolated in gut samples from this study, has been associated with skin and soft tissue infections in humans (McGhie et al., 2012). Rhodococcus genera can be found in various sources and many species are implicated in human diseases (Majidzadeh & Fatahi-Bafghi, 2018). However, the species presented in this study, *Rhodococcus degradans* and *R. fascians* have only been associated with biodegradation capabilities (Svec et al., 2015) and diseases in plants (Dhaouadi & Rhouma, 2020). To our best knowledge, their association with shrimp gut microbiota has not been described, although the Nocardiaceae family has been previously reported in the intestinal bacterial community of Pacific white shrimp (Cheng et al., 2021).

From all the genera present exclusively in the CTRL group, Flavobacterium genus is known to include pathogenic species for fish and shrimp (Bernardet et al., 1996; Sivakamavalli et al., 2021). When analysing the individual OTUs from this genus, two species emerged: *Flavobacterium oncorhynchi*, previously reported as isolated from rainbow trout (Zamora et al., 2012) and *Flavobacterium haoranii* firstly described in a freshwater shrimp culture pond (Sheu et al., 2013). Prevotella genus was also identified solely in the CTRL group and is one of the main enterotypes observed in human microbiomes (Tomova et al., 2019). This genus protects the intestine with its anti-inflammatory properties, and has been associated with plant-rich, low fat and low animal protein diets, in humans (Tomova et al., 2019). Lactococcus genus has been studied as probiotic in *Penaeus vannamei* (Adel et al., 2017), however the species *Lactococcus garvieae* and *L. lactis* subspecies *lactis* can be pathogenic for the giant freshwater prawn *Macrobrachium rosenbergii* (Wang et al., 2008; Adel et al., 2017). In this study, only the species *Lactococcus cremoris* was identified. Pediococcus was also found exclusively in the CTRL group, represented by the species *Pediococcus pentosaceus*. This species has been evaluated has a probiotic in shrimp aquaculture, improving the weight gain, growth rate and digestive enzyme activities (Wanna et al., 2021).

Regarding genera only exclusively found in the KRILL group, there is Halobacteriovorax, a predatory bacterium, that contributes to bacterial death, and is associated with mortality and reduced density of *Vibrio parahaemolyticus* populations (Williams et al., 2016). The Lactobacillus genera, found also solely in the KRILL group, is commonly associated to beneficial bacteria and a well-known probiotic used in both humans and animal species (Gaucher et al., 2019). Lactobacillus possesses adaptation mechanisms to respond to environmental conditions and modulates the gut microbiota, stimulating beneficial bacteria and inhibiting pathogens growth (Gaucher et al., 2019). The presence of both Halobacteriovorax and Lactobacillus exclusively on the KRILL group might indicate a modulation property of the KRILL group towards beneficial bacteria. Similar studies are needed to fully corroborate this hypothesis and understand the effects of KM and KO in the Pacific white shrimp intestinal microbiota.

5. Conclusions

The findings of this study showed that KM and KO allowed a 50% replacement of FM and FO in a high soybean meal-based diet without affecting the overall growth performance and systemic innate immunity of Pacific white shrimp juveniles. However, while the overall intestinal microbiota was not significantly affected by the dietary regimes, shrimps seem to benefit from the inclusion of KM and KO, with the presence of beneficial bacteria that control bacterial populations and promote intestinal health.

6. Acknowledgements

This work was partially supported by Projects FICA (reference ALG-01-0247-FEDER-047175), UIDB/04423/2020 and UIDP/04423/2020, financed by Portugal and the European Union through FEDER, COMPETE 2020, and CRESC Algarve 2020, in the framework of PORTUGAL2020, and through the COMPETE and Operational Human Potential Programs and national funds through Fundação para a Ciência e a Tecnologia (FCT, Portugal). C. Teixeira and B. Costas were supported by FCT, Portugal (PD/BDE/135541/2018 and 2020.00290.CEECIND, respectively). C. Teixeira was also supported by SPAROS through grant PD/BDE/135541/2018. We thank Professor Alicia Toranzo for kindly providing the bacterial strain (*Vibrio harveyi*; IA20.2) used in this study and to Fátima Faria for monitoring the histological processing.

7. References

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Chapter VI

General Discussion, Conclusions and Future perspectives

General discussion, Conclusions and Future perspectives

1. General discussion

The main objective of this PhD thesis was to study how different factors (i.e., antibiotics, diets, inflammation) can modulate intestinal health, and subsequently to understand if it can influence fish health. As the intestine of fish and shrimp is very prone to manipulation through diet, and intestinal health can produce systemic effects, an holistic approach was taken; firstly, to address the effects of several chemicals and ingredients on intestinal health; secondly, to evaluate the impact of feeds as a mechanism to ameliorate and/or restore the intestinal health; and thirdly, to assess the effects of different additives in the intestinal health.

1.1. Deleterious effects of chemicals and vegetable ingredients in intestinal health

Intestinal inflammation can be caused by a combination of different factors, such as genetic susceptibility, dysbiosis, inappropriate immune system activity, and environmental agents. Chemicals, diets, stress, and pathogens can be used in larval and adult fish and shrimp as a mechanism to induce and study intestinal inflammation. To evaluate the dietary effects on intestinal health, two trials were conducted. The first, in Nile tilapia (**Chapters II** and **III**), where an antibiotic was used as a chemical stressor; and the second (**Chapter IV**), in gilthead seabream, where the DSS was used also as an inducer of gut inflammation.

As described by previous authors, the use of chemicals to induce intestinal damage is dose- and time-dependent. For instance, the antibiotic dose (oxytetracycline) used in **Chapters II** and **III**, affected the intestinal gene expression profile, modified the oxidative stress response, and altered the dynamics of the intestinal microbiota of Nile tilapia, however, was not sufficient to induce significant damage on the intestinal wall and produce noteworthy systemic effects. Similar results happened in **Chapter IV**, where the chemical DSS used to induce intestinal inflammation in gilthead seabream, modified the intestinal gene expression profile and stimulated a redox response on the intestinal wall, however, it was not able to produce a significant inflammatory response.

It is also important to retain, that the damage induced in the intestinal structure and associated microbiota can generate a systemic response, as seen in **Chapters II** and **IV**, by the antibiotic- or DSS-related manifestations on Nile tilapia or gilthead seabream haematology and immunology. However, it was verified that such manifestations were not sufficient to affect fish growth. Longer periods of exposition or higher doses seem to be necessary to produce more severe damage to the microbiota community and the intestinal structure of fish, as well as, to induce a profound influence on the fish's health. At least for the species under study.

As described before, diets can also be used to induce intestinal inflammation through the presence of higher amounts of fibre and/or anti-nutritional factors. As seen in **Chapters II** and **III**, the high plant-based diet induced an adjustment in the intestinal microbial community and the intestinal wall and affected some immunological and haematological parameters through their dietary components. The synergy of the antibiotic with the high plant-based diet was the only stressor able to directly affect growth.

1.2. Functional diets as recovery mechanisms for intestinal damage

As mentioned before, the intestine of fish and shrimp is very prone to manipulation through diet, and diets can be used as a recovery mechanism to retrieve and improve intestinal health after an injury. In **Chapters II**, **III**, and **IV**, with Nile tilapia and gilthead seabream, it was possible to evaluate the restorative capabilities of different diets in the intestinal and overall health of fish.

Despite the absence of severe intestinal disease present in **Chapters II**, **III**, and **IV**, after the chemical-induced damages, diets proved to be ameliorative mechanisms to the adverse effects generated by the previous chemical stressors. In **Chapters II** and **III**, it was possible to verify that the recovery diets were able to minimize the antibiotic negative effects on the microbiota community, by almost 50%. The high plant-based diet was more successful in the modulation and recovery of the dynamics of the microbiota community to a new homeostatic state than the control diet; however, it induced some deleterious effects on the fish's oxidative-stress response, immunity, haematology, and growth.

Likewise, in **Chapter IV**, the feed additives were able to ameliorate most of the DSS-induced modifications on the intestinal gene expression profile and oxidative stress response in that organ, being also able to counteract some of the DSS deleterious effects in the haematological profile. The known biological properties present in these additives were important to minimize the DSS effects.

1.3. Functional diets as intestinal health enhancers

As described before, several ingredients and nutrients can be used to improve a diet's nutritional value. In **Chapter IV**, it was possible to study the effects of two feed additives (*Phaeodactylum tricornutum* extract rich in β-glucan and curcumin) supplemented to diets for gilthead seabream as a recovery strategy to minimize the adverse effects of gut inflammation and, in **Chapter V** it was evaluated the fishmeal and fish oil replacement by krill meal and oil in a shrimp diet.

Previous authors highlighted the importance of using functional feeds to improve fish health. As verified in **Chapter IV**, the two feed additives used were able to modify some of the inflammatory effects of the DSS; however, such manifestations were not significant in the absence of a stimulus. Therefore, it is important to have in mind that the beneficial improvements of feed additives are normally expressed in the presence of stressors and can be absent or moderated in normal non-stressed conditions. Consequently, the use of functional feeds, due to the increased associated costs, should be used as preventing measures to challenging situations, such as handling, transport, and temperature variations.

As described previously, the replacement of fishmeal and fish oil can produce some deleterious effects on the intestinal and overall health of fish and shrimp, however, as seen in **Chapter V** in the whiteleg shrimp, no major detrimental effects were observed after the replacement of fishmeal and fish oil by krill meal and triglyceride-rich krill oil in the diet. Krill is still a high-cost ingredient, however, improvements in production and transport can reduce its production costs and balance its known biological properties with its associated dietary cost.

2. Conclusions

The main conclusions of this Thesis are:

1 - The recommended dose of oxytetracycline in Portugal in a dietary treatment (55 mg Kg^{-1} body weight day^{-1}, 10 days) is relatively safe to be used in aquaculture since originated only mild variations in the intestinal and overall health of healthy fishes (**Chapters II** and **III**);

2 - Commercial and high plant-based diets are able to minimize the deleterious effects of the intestinal inflammatory conditions induced by oxytetracycline, however, high plant-based diets can impair a complete recovery even in omnivorous species (**Chapters II** and **III**);

3 - Chemicals such as DSS can be used as a model to study the intestinal inflammatory process, however, the inflammation is dose and time-dependent and the grade of inflammation needs to be fully established to better understand the recovery process (**Chapter IV**);

4 - Functional diets (with *Phaeodactylum tricornutum* β-glucan and curcumin) can be used as health enhancers, however their effect may not be significant in the absence of a stressor, and they should be used previously to a stress event (**Chapter IV**).

5 - Feed additives, such as *Phaeodactylum tricornutum* β-glucan and curcumin, are able to minimize the intestinal inflammatory effects induced by DSS, however, their concentration and their associated cost should be addressed (**Chapter IV**);

6 - Alternative ingredients, such as, krill can replace fishmeal and fish oil, however, their concentration and their associated cost should be addressed and balanced with their beneficial effects (**Chapter V**);

3. Future approaches

This Thesis addressed the need to improve intestinal health in fish and shrimp through diets, with a focus on the inflammatory condition, immune status, and microbiota diversity. Nonetheless, much more research is needed to fully understand the deleterious impacts of stressors on the intestinal microbiota and intestinal structure as well as immune responses.

Although this Thesis helped to understand some of the associated effects of intestinal chemical stressors, such as oxytetracycline (**Chapters II** and **III**) and DSS (**Chapter IV**), dose and time remain to be established as a model to study dysbiosis and intestinal inflammation, respectively. Moreover, it also remained to be established a model to study the effect of an extreme diet as an inducer of intestinal inflammation in Nile tilapia, which would be useful in future studies with functional diets. Nevertheless, it is important to keep in mind that the high-plant diet (**Chapters II** and **III**) was used to evaluate recovery in an extreme scenario and not as an inflammatory inductor.

Also, a bacterial challenge can be used to better understand the benefits of feed additives (such as those used in **Chapter IV**), or the consequences of fishmeal and fish oil replacement by alternative ingredients (such as krill used in **Chapter V**), since the biological activities of feed additives are expressed in the presence of stressors, and the replacement of fishmeal and fish oil can impair the immunological response towards pathogens.

Appendix

Appendix 1

Modulation of intestinal morphology and microbiota diversity by the dietary administration of oxytetracycline in Nile tilapia (*Oreochromis niloticus*)

Supplementary Table S1. MiSeq general sequencing data obtained in the anterior (A) and posterior (P) intestinal samples of Nile Tilapia fed with the experimental diets (C - control diet, O - oxytetracycline diet, OC - oxytetracycline + control diet, OP - oxytetracycline + plant diet).

Anterior Intestine									
Day	Diet	SampleID	Total Bases	Read Count before pre-processing	Read Count after Clustering (97% cutoff)	GC (%)	Q20 (%)	Q30 (%)	OTUs
10	C	TA4	37259694	83072	24400	53,47	97,75	92,13	189
10	C	TA5	45259861	99208	41131	52,3	97,98	92,78	345
10	C	TA6	30479969	68613	13080	51,31	98,05	92,79	185
10	O	TA7	44237925	98263	29076	51,92	97,85	92,56	281
10	O	TA8	50274806	111629	29605	51,59	97,89	92,64	242
18	C	TA16	48156829	106298	35629	53,32	97,77	92,27	342
18	C	TA17	36412902	81193	20398	52,11	97,9	92,57	375
18	C	TA18	46467580	103115	30080	53,68	97,65	91,89	383
18	OC	TA19	45176926	99323	36284	53	97,86	92,53	405
18	OC	TA20	50367696	111364	39452	51,97	97,79	92,38	320
18	OC	TA21	36088838	80012	22073	52,19	97,8	92,37	344
18	OP	TA22	42220903	93748	26736	52,2	97,85	92,39	256
18	OP	TA23	45996902	101042	35759	53,49	97,63	92,04	313
25	C	TA24	39009382	86063	28183	54,21	97,65	92,02	279
25	C	TA25	53980410	116801	39705	54,36	97,59	91,93	299
25	OC	TA26	45215689	99537	35200	53,8	97,62	91,86	298
25	OC	TA28	44015647	97386	30523	53,49	97,87	92,49	317
25	OP	TA29	44579965	98795	31976	53,8	97,96	92,66	323
25	OP	TA30	34384652	75362	29738	53,53	97,71	92,2	169
40	C	TA31	36114251	79972	24696	52,96	97,73	92,1	225
40	C	TA32	18098642	40235	12108	53,12	97,73	92,2	51
40	OC	TA33	44147243	96627	38701	52,82	97,7	92,14	231
40	OC	TA34	50146073	109193	49653	52,19	97,77	92,39	251
40	OP	TA35	46327953	101693	34740	53,21	97,79	92,3	445
40	OP	TA36	52693729	113858	62179	52,36	97,35	91,08	180

Posterior Intestine									
Day	Diet	SampleID	Total Bases	Read Count before pre-processing	Read Count after Clustering (97% cutoff)	GC (%)	Q20 (%)	Q30 (%)	OTUs
10	C	TP4	38452304	84596	29351	52,12	97,9	92,63	419
10	C	TP5	34190647	76176	25667	52,88	97,91	92,53	257
10	C	TP6	36677062	81905	26484	51,94	97,94	92,63	187
10	O	TP7	41623149	91195	40587	49,6	97,98	92,82	94
10	O	TP8	41260105	91057	27526	50,08	97,92	92,67	169
18	C	TP18	30014921	67022	17931	51,63	98,03	92,82	183
18	C	TP19	30281847	67765	20346	52,91	97,88	92,43	160
18	C	TP20	34440902	76921	22272	51,5	97,99	92,75	229
18	OC	TP21	39686117	88110	30696	50,39	97,93	92,68	134
18	OC	TP22	31854954	70212	27800	52	97,87	92,53	182
18	OC	TP23	48407854	107456	30443	52,17	97,86	92,47	416
18	OP	TP24	47544268	105342	36236	53,15	97,78	92,33	322
18	OP	TP25	48949442	107041	38077	52,56	97,73	92,25	436
18	OP	TP26	34156748	76470	19599	51,77	98,05	92,89	210
25	C	TP27	34679042	77847	17130	50,83	98,01	92,76	197
25	C	TP28	34118695	76104	20447	51,44	98,06	92,97	233
25	OC	TP30	45026641	99049	37489	51,6	97,93	92,72	253
25	OC	TP31	47631423	105993	27033	52,85	97,74	92,22	290
25	OC	TP32	50361160	111782	34702	51,08	98	92,84	286
25	OP	TP34	52955971	117353	36116	53,05	97,85	92,46	400
25	OP	TP35	46980725	103849	24095	53,94	97,78	92,22	500
40	C	TP36	47102190	104621	35417	51,98	97,92	92,71	305
40	C	TP37	35410066	78479	29273	51,71	97,8	92,36	124
40	C	TP38	35885218	79998	17430	51,85	98,04	92,89	313
40	OC	TP39	50529242	110809	33776	52,98	97,73	92,24	490
40	OC	TP40	39891593	87148	32097	52,19	97,85	92,51	388
40	OC	TP41	34120268	75136	21622	52,5	97,77	92,28	384
40	OP	TP42	32332077	71456	25408	53,13	97,82	92,29	360
40	OP	TP43	40732251	90725	27374	52,36	97,88	92,52	348
40	OP	TP44	41832902	92826	27848	53,19	97,88	92,5	362

Supplementary Table S2. Fold-change analysis from the ecological parameters obtained from NGS analysis of the Nile tilapia anterior and posterior intestinal microbiota after the dietary medicated treatment with oxytetracycline for 10 days. O (oxytetracycline diet), OC (oxytetracycline + control diet), OP (oxytetracycline + plant diet). Evaluation of the direct effects (day 10; O/C) and recovery (days 18, 25 and 40; OC/O or OP/O). The asterisk represents significant differences between the antibiotic and the control diet ($p < 0.05$).

		Anterior							Posterior						
		10 O	18 OC	25 OC	40 OC	18 OP	25 OP	40 OP	10 O	18 OC	25 OC	40 OC	18 OP	25 OP	40 OP
Genus	Akkermansia	↓	↑	↓	↓	↑	↑	↑	↓			↓	↑	↑	↑
	Cetobacterium	↑	↓	↓	↓	↓	↓	↓	↓	↑	↑	↓	↑	↓	↑
	Bacteroides	↑	↑	↓	↓	↓	↓	↓	↑*	↓	↓	↓	↓	↓	↓
	Ligilactobacillus	↓	↑	↑	↑	↑	↓	↓	↓	↑	↑	↑	↑	↑	↑
	Legionella	↑*	↑	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↑
	Secundilactobacillus	↓	↓	↑	↑	↑	↑	↓	↓	↑	↑	↑	↑	↑	↑
	Luteolibacter	↓	↑	↑	↑	↑	↑	↑	↓	↑	↑	↑	↑	↑	↑
	Leifsonia	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
	Parabacteroides	↓	↑	↓	↓	↓	↓	↓	↑	↓	↓	↓	↓	↓	↓
	Romboutsia	↓	↑	↑	↑	↑	↑	↑	↓						
	Pseudomonas	↑	↓	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↑
	Klebsiella	↑	↓	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↑
	Aerosakkonema	↑	↑	↑	↓	↓	↑	↓	↓	↑	↑	↑	↑	↑	↑
	Ralstonia	↑	↓	↑	↑	↑	↑	↓	↓	↑	↑	↑	↑	↑	↑
	Paludibacter	↓							↑	↓	↓	↓	↓	↓	↓
	Plesiomonas	↓							↑	↑	↓	↓	↓	↓	↓
	Gloeobacter	↑	↓	↑	↓	↑	↑	↓	↓	↑	↑	↑	↑	↑	↑
	Candidatus Protochlamydia	↓	↑	↑	↑	↑	↑	↑	↓	↑	↑	↑	↑	↑	↑
	Hyphomicrobium	↑	↓	↓	↑	↑	↑	↓	↓	↑	↑	↑	↑	↑	↑
	Hydrobacter	↑	↓	↓	↓	↑	↑	↓	↓	↑	↑	↑	↑	↑	↑
	Nocardioides	↓	↑	↑	↑	↑	↑	↑	↓	↑	↑	↑	↑	↑	↑
	Parachlamydia	↑	↓	↓	↑	↓	↓	↑	↓	↑	↑	↑	↑	↑	↑
	Neochlamydia	↑	↓	↑	↓	↑	↓	↓	↓	↑	↑	↑	↑	↑	↑
	Simkania	↓	↓	↑	↓	↑	↑	↑	↓	↑	↑	↑	↑	↑	↑
	Fimbriiglobus	↓	↓	↑	↑	↑	↑	↑	↓						
	Bosea	↑	↓	↓	↓	↓	↓	↓	↓	↑	↓	↑	↑	↑	↑
	Lysinimonas	↑	↑	↓	↓	↑	↓	↓	↓	↑	↑	↑	↑	↑	↑
	Coxiella	↑	↓	↑	↓	↑	↓	↑	↓	↑	↑	↑	↑	↑	↑
	Acinetobacter	↑	↓	↑	↑	↑	↓	↓	↓	↑	↑	↑	↑	↑	↑
	Pirellula	↓							↓	↑	↑	↑	↑	↑	↑
	Sediminibacterium	↑	↓	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↑
	Rhodobacter	↓							↓	↑	↑	↑	↑	↑	↑
	Marinobacter	↑	↓	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↑
	Bradyrhizobium	↑	↑	↑	↑	↑	↑	↓	↓	↑	↑	↑	↑	↑	↑
	Caedimonas	↓	↑	↑	↑	↑	↑	↑	↓	↑	↑	↑	↑	↑	↑
	Curvibacter	↑	↓	↓	↑	↑	↑	↓	↓	↑	↑	↑	↑	↑	↑
	Chlamydia	↓	↓	↑	↓	↓	↓	↓	↓						
	Aquihabitans	↑	↓	↓	↓	↑	↓	↓	↓	↑	↑	↑	↑	↑	↑
	Conexibacter	↓	↑	↑	↑	↑	↑	↑	↓						
	Bacillus	↑*	↓	↓	↓	↑	↓	↓	↑	↑	↑	↑	↑	↑	↑
	Corynebacterium	↓	↓	↑	↑	↑	↑	↑	↓	↑	↑	↑	↑	↑	↑
	Rhodanobacter	↑	↓	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↑
	Sulfuriflexus	↓	↓	↑	↓	↑	↑	↓	↓	↑	↑	↑	↑	↑	↑
	Thermostilla	↑	↓	↓	↓	↑	↓	↓	↓	↑	↑	↑	↑	↑	↑
	Fusobacterium	↓	↓	↑	↑	↑	↑	↑	↓	↑	↑	↑	↑	↑	↑
	Leucobacter	↑*	↓	↓	↓	↓	↓	↓	↑	↓	↓	↓	↓	↓	↓
	Mesorhizobium	↑	↓	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
	Rhodococcus	↓	↓	↑	↓	↑	↑	↓	↓						
	Cloacibacterium	↑	↓	↓	↓	↓	↓	↓	↓						
	Aeromicrobium	↑*	↑	↑	↓	↑	↑	↓	↓	↑	↑	↑	↑	↑	↑
	Brevundimonas	↑	↓	↑	↑	↓	↑	↓	↑	↑	↑	↑	↑	↑	↑
	Turicibacter	↓							↓						
	Vibrio	↑	↓	↓	↓	↓	↑	↓	↓	↑	↑	↑	↑	↑	↑
	Gemmata	↓	↑	↑	↑	↑	↑	↑	↓						
	Minicystis	↓	↓	↓	↑	↑	↑	↓	↓	↑	↑	↑	↑	↑	↑
	Ensifer	↓							↓	↓	↓	↓	↓	↑	↑
	Sphingomonas	↑	↓	↓	↓	↑	↑	↓	↓	↑	↑	↑	↑	↑	↑
	Litorilinea	↓							↓						
	Staphylococcus	↑	↓	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↑
	Streptococcus	↑	↓	↑	↑	↓	↓	↑	↑	↑	↑	↑	↓	↑	↑
	Flavihumibacter	↓							↓						
	Limnoglobus	↓							↓						
	Chondromyces	↑	↓	↑	↓	↑	↑	↓	↓						
	Aeromonas	↓	↓	↓	↓	↑	↓	↓	↓	↓	↑	↓	↑	↑	↑
	Lactiplantibacillus	↓							↓						
	Micrococcus	↑	↓	↓	↓	↑	↑	↓	↓						
	Paucibacter	↓							↓						
	Kocuria	↓*	↓	↑	↓	↓	↑	↓	↓						
	Levilactobacillus	↓	↓	↓	↓	↓	↓	↓	↓						
	Brachybacterium	↓							↓	↓	↑	↑	↑	↑	↓
	Massilia	↑	↓	↑	↓	↑	↓	↓	↓						
	Lactobacillus	↑	↓	↓	↓	↓	↓	↓	↓						
	Alkanindiges	↓	↓	↑	↓	↓	↑	↑	↓						
	Rossellomorea	↓							↓	↓	↓	↓	↓	↓	↓
	Dorea	↓							↓						
	Marivita	↓	↓	↓	↓	↓	↓	↓	↓						
	Prevotella	↓	↓	↓	↓	↓	↓	↓	↓						

Recovery

No recovery

Appendix 2

Dietary strategies to modulate the health condition and immune responses in gilthead seabream (*Sparus aurata*) juveniles following intestinal inflammation

Supplementary Table S1. Intestinal gene expression profiles of gilthead seabream after the feeding and inflammatory trials. **CTRL** (control diet), **BG** (β-glucans diet), **CUR** (curcumin diet), **CTRL-D** (control diet + dextran sodium sulphate - DSS), **BG-D** (β-glucans diet + DSS), **CUR-D** (curcumin diet + DSS). Values are presented as means ± standard deviation. Different letters mean significant differences among dietary treatments ($p < 0.05$).

Gene expression	Feeding trial			Inflammatory trial			
	CTRL	BG	CUR	CTRL	CTRL-D	BG-D	CUR-D
sod	0.0383 ± 0.0150	0.0412 ± 0.0161	0.0367 ± 0.0218	0.0434^{a} ± 0.0162	0.0294^{ab} ± 0.0200	0.0197^{b} ± 0.0104	0.0333^{ab} ± 0.0161
gpx	0.0108 ± 0.0060	0.0111 ± 0.0056	0.0110 ± 0.0086	0.0049 ± 0.0040	0.0033 ± 0.0023	0.0024 ± 0.0012	0.0037 ± 0.0023
tnfα	0.0033^{b} ± 0.0009	0.0039^{ab} ± 0.0009	0.0060^{a} ± 0.0036	0.0034 ± 0.0020	0.0019 ± 0.0010	0.0028 ± 0.0021	0.0020 ± 0.0016
il10	0.0001 ± 0.0001	0.0001 ± 0.0001	0.0001 ± 0.0000	0.0001 ± 0.0001	0.0001 ± 0.0000	0.0000 ± 0.0000	0.0001 ± 0.0000
igm	0.0581 ± 0.0593	0.0352 ± 0.0290	0.0416 ± 0.0649	0.0537 ± 0.0424	0.0232 ± 0.0116	0.0255 ± 0.0160	0.0261 ± 0.0225
csf1r	0.0002^{b} ± 0.0001	0.0003^{ab} ± 0.0002	0.0004^{a} ± 0.0002	0.0003^{a} ± 0.0001	0.0002^{b} ± 0.0001	0.0002^{ab} ± 0.0001	0.0002^{ab} ± 0.0001
cd8α	0.0141 ± 0.0089	0.0121 ± 0.0056	0.0112 ± 0.0050	0.0131 ± 0.0115	0.0060 ± 0.0020	0.0075 ± 0.0037	0.0088 ± 0.0046
hsp70	0.2583 ± 0.0703	0.3213 ± 0.0886	0.4105 ± 0.2658	0.3349 ± 0.2583	0.1604 ± 0.0711	0.2074 ± 0.1460	0.2976 ± 0.1910
ocln	0.0146 ± 0.0045	0.0233 ± 0.0082	0.0242 ± 0.0157	0.0116 ± 0.0092	0.0068 ± 0.0050	0.0060 ± 0.0036	0.0089 ± 0.0044
hep	0.3523^{b} ± 0.2303	0.6998^{ab} ± 0.4985	1.0486^{a} ± 0.7749	0.3716 ± 0.3151	0.4004 ± 0.3432	0.1642 ± 0.1878	0.3237 ± 0.2378
muc13	0.2820 ± 0.1098	0.2894 ± 0.1285	0.2659 ± 0.1237	0.2385 ± 0.1451	0.1520 ± 0.1272	0.1766 ± 0.1094	0.1776 ± 0.0908
muc2	0.0488 ± 0.0190	0.0482 ± 0.0229	0.0447 ± 0.0312	0.0260 ± 0.0173	0.0108 ± 0.0059	0.0220 ± 0.0138	0.0207 ± 0.0107

Appendix 3

Effect of krill meal and a triglyceride-rich krill oil on the immunity and intestinal microbiota of the Pacific white shrimp (*Penaeus vannamei*) fed a high-soybean meal diet

Supplementary Table S1. MiSeq general sequencing data obtained from intestinal samples of Pacific white shrimp, fed the experimental diets CTRL (control diet) and KRILL (krill diet).

Diet	Sample name	SampleID	Total Bases	Read Count before pre-processing	Read Count after Clustering (97% cutoff)	GC (%)	Q20 (%)	Q30 (%)	OTUs	Chao1	Shannon	Simpson	GoodsCoverage
CTRL	CTRL-1	CTRL1	47 175 031	101965	42818	52,67	97,66	92,26	125	134,333333	2,8842707	0,78062199	0,999830842
CTRL	CTRL-2	CTRL2	46 149 527	99430	51019	52,86	97,79	92,56	39	44	1,07912898	0,3604436	0,999883011
CTRL	CTRL-3	CTRL3	50 072 716	108168	46154	52,42	97,67	92,21	83	91,0769231	2,50349281	0,77408695	0,999681846
CTRL	CTRL-4	CTRL4	50 773 059	109743	49639	52,88	97,67	92,24	93	97,0909091	2,15490035	0,71508112	0,99979943
CTRL	CTRL-5	CTRL5	41 472 097	89465	44690	52,82	97,65	92,26	52	70,3333333	1,94855546	0,65965915	0,999755044
KRILL	KRILL-1	KRILL1	52 962 093	114452	38337	52,73	97,88	92,79	102	106,4	2,87010168	0,79044214	0,999713289
KRILL	KRILL-2	KRILL2	44 853 534	96714	41781	52,61	97,59	92,06	78	85,1578947	1,46473972	0,47204334	0,999595171
KRILL	KRILL-3	KRILL3	47 850 480	103325	41703	52,79	97,73	92,39	100	108,5	2,53007249	0,77166929	0,999599699
KRILL	KRILL-4	KRILL4	40 197 133	86580	42859	52,97	97,74	92,52	53	54	2,35493498	0,74368484	0,999884457
KRILL	KRILL-5	KRILL5	47 989 156	103509	50906	52,78	97,82	92,61	38	42,6666667	2,02345416	0,70843013	0,999842956