

DOUTORAMENTO EM CIÊNCIA ANIMAL
MORFOLOGIA E FISIOLOGIA

Boosting fish robustness: Unveiling the potential of algae feeds for enhanced intestinal health and performance

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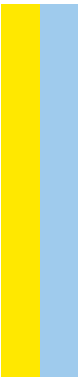


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Fundação
para a Ciência
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Diretivas Legais

No cumprimento do disposto no Decreto-Lei no 204/2018 de 23 de Outubro, declara-se que o autor desta tese participou na conceção e na execução do trabalho experimental que esteve na origem dos resultados apresentados, bem como na sua interpretação e na redação dos respetivos manuscritos.

Nesta tese incluem-se ainda quatro artigos científicos publicados e um artigo submetido em revistas internacionais resultantes de uma parte dos resultados obtidos no trabalho experimental, referenciados como:

Ferreira, M., Teixeira, C., Abreu, H., Silva, J., Costas, B., Kiron, V., & Valente, L. M. P. (2021). Nutritional value, antimicrobial and antioxidant activities of micro- and macroalgae, single or blended, unravel their potential use for aquafeeds. *J. Appl. Phycol.*, 33, 3507–3518. <https://doi.org/10.1007/s10811-021-02549-2>

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Legal Details

In compliance with what is stated in Decree-Law no. 204/2018 of 23 October 23, it is hereby declared that the author of this thesis participated in the creation and execution of the experimental work leading to the results shown, as well as in their interpretation and the writing of respective manuscripts.

This thesis includes four scientific papers published and one submitted in international journals from part of the results obtained in the experimental work, referenced to as:

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"Mistakes... is the word you're too embarrassed to use. You ought not to be. You're a product of a trillion of them. Evolution forged the entirety of sentient life on this planet using only one tool: the mistake" in Westworld.

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List of abbreviations

AA - Amino Acids

AB/PAS - Alcian Blue/Periodic Acid Schiff

ACH50 - Alternative Complement Pathway Haemolytic Activity

a2m - Alpha-2-macroglobulin

alp - Alkaline phosphatase

aqp1 - Aquaporin 1

ARA - Arachidonic Acid

ASVs - Amplicon Sequence Variants

βactin - Beta actin

CAGR - Compound Annual Growth Rate

cat - Catalase

c1q4 - Complement Component 1q Subcomponent 4

c3 - Complement Component 3

c9 - Complement Component 9

casp3 - Caspase 3

casp9 - Caspase 9

ccr9 - C-C Chemokine Receptor Type 9

cd4 - Cluster of Differentiation 4

cd3ε - T-Cell Surface Glycoprotein CD3 Epsilon

cd33 - Cluster of Differentiation 33

cd8α - T-Cell Surface Glycoprotein CD8 Alpha Chain

CEAA - Conditionally Essential Amino Acids

CHLO - *Chlorella vulgaris*

cld1 - Claudin 1

CSP - Cross-Sectional Perimeter

CTRL - Control

DHA - Docosahexaenoic Acid

dic - Dicentracin

defβ - Defensin Beta

DTT - Dithiothreitol

EAA - Essential Amino Acids

EDTA - Ethylenediamine Tetraacetic Acid

ef1α - Elongation Factor 1 Alpha

EPA - Eicosapentaenoic Acid

fabp2 - Intestinal Fatty Acid Binding Protein 2

FACS - Fluorescence-Activated Cell Sorting
FBW - Final Body Weight
FBS - Foetal Bovine Serum
FCR - Feed Conversion Ratio
FM - Fish Meal
FO - Fish Oil
FS - Forward Scatter
FVD - Fixable Viability Dye
GALTs - Gut-Associated Lymphoid Tissues
GC - Goblet Cells
GI - Gastro-Intestinal
GRA - *Gracilaria gracilis*
GSH - Glutathione
GPx - Glutathione Peroxidase
grp-75 - Glucose-Regulated Protein 75
grp-78 - Glucose-Regulated Protein 78
grp-94 - Glucose-Regulated Protein 94
grp-170 - Glucose-Regulated Protein 170
HBSS - Hank's Balanced Salt Solution
Hb - Haemoglobin
hep - Hepcidin Precursor
Ht - Haematocrit
IBW - Initial Body Weight
IFC - Imaging Flow Cytometry
IgG - Immunoglobulin G
IgM - Immunoglobulin M
IgT - Immunoglobulin T
igt - Insulin-Like Growth Factor Transcript
il-1 β - Interleukin-1 Beta
il-6 - Interleukin-6
il-8 - Interleukin-8
il-10 - Interleukin-10
il-9 - Interleukin-9
il-34 - Interleukin-34
IMTA - Integrated Multitrophic Aquaculture
LEL - *Lycopersicon esculentum* (Tomato) Lectin
malt - Maltase

MALTs - Mucosa-Associated Lymphoid Tissues
 MB - Marine Broth
 MCH - Mean Corpuscular Haemoglobin
 MCHC - Mean Corpuscular Haemoglobin Concentration
mcsfr1 - Macrophage Colony-Stimulating Factor Receptor 1
 MCV - Mean Corpuscular Volume
 MDA - Malondialdehyde
 MGG - May Grünwald-Giemsa
mif - Macrophage Migration Inhibitory Factor
mnsod - Manganese Superoxide Dismutase
 MUC2 - Mucin 2
 MUFA - Monounsaturated Fatty Acids
myd88 - Myeloid Differentiation Primary Response 88
 NAN - *Nannochloropsis oceanica*
 NANGRA - Blend of *G. gracilis* and *N. oceanica*
 NEAA - Nonessential Amino Acids
nf-2 - Neurofibromin 2
ocln - Occludin
 PBS - Phosphate Buffer Saline
 PCA - Principal Component Analysis
pcna - Proliferating Cell Nuclear Antigen
Phdp - *Photobacterium damsela* subspecies *piscicida*
pisc1 - Piscidine 1
p65 - Nuclear Factor NF-Kappa-B p65 Subunit
 PP - Plant Protein
 LC-PUFAs - Long-Chain Polyunsaturated Fatty Acids
 RaRBC - Rabbit Red Blood Cells
rag-1 - Recombination Activating Gene 1
 RBC - Red Blood Cells
 RT - Room Temperature
 RT-PCR - Real-Time Reverse Transcription-Polymerase Chain Reaction
 SFA - Saturated Fatty Acids
 SGR - Specific Growth Rate
sod - Superoxide Dismutase
 SS - Side Scatter
tcrl - T-Cell Receptor Antigen Receptor Beta Chain
tgfb - Transforming Growth Factor Beta

tlr9 - Toll-like Receptor 9

tnf- α - Tumor Necrosis Factor Alpha

tric - Triosephosphate Isomerase

ULVA - *Ulva rigida*

WBC - White Blood Cells

WGA - Wheat Germ Agglutinin

zo-1 - Zonula Occludens-1

Resumo

À medida que a população mundial continua a crescer, o setor da aquacultura vê-se confrontado com o desafio de satisfazer a crescente procura por peixe. O sistema intensivo de produção de peixes resulta frequentemente em surtos de doenças, estando estes associados a elevadas taxas de mortalidade e a grandes perdas económicas. Deste modo, o desenvolvimento de abordagens inovadoras para fortalecer a resistência e o bem-estar dos peixes está a tornar-se cada vez mais importante, especialmente em resposta aos desafios colocados pelo aumento das resistências antimicrobianas e o impacto destas na sustentabilidade do setor da aquacultura. Neste contexto, os potenciais benefícios da inclusão de algas nas dietas de peixes começaram a ser explorados, com o objetivo de encontrar soluções baseadas em recursos naturais, para melhorar a saúde dos peixes, reduzir a dependência de antibióticos e responder à tendência para uma alimentação saudável, associada à ética de consumo.

Na fase inicial deste projeto de doutoramento foi realizada uma análise detalhada de espécies de algas disponíveis comercialmente, de forma a revelar o seu potencial bio-funcional: microalgas *Nannochloropsis oceanica* e *Chlorella vulgaris* e macroalgas *Gracilaria gracilis* e *Ulva rigida*. De modo a explorar o potencial coletivo destas biomassas, uma combinação destas algas foi também testada. Após a determinação das suas propriedades químicas e funcionais, estas algas foram incorporadas individualmente ou em combinação em dietas para robalo Europeu (*Dicentrarchus labrax*) de forma a avaliar o seu potencial efeito na microbiota e na estrutura intestinal. Foi também realizado um ensaio de crescimento utilizando rações com a incorporação de algas, seguido de um ensaio de infeção, de forma a obter mais informações sobre o potencial destas algas em aumentar a resistência do peixe durante um surto de doença, após exposição ao agente patogénico *Tenacibaculum maritimum*. De forma a perceber a eficácia das rações testadas, as taxas de mortalidade e as respostas imunes ao nível sistémico e das mucosas foram avaliadas através da análise do perfil hematológico, parâmetros imunológicos no plasma e padrões de expressão genética nos tecidos da pele, intestino e rim.

Os resultados obtidos demonstraram que tanto as microalgas (*N. oceanica* e *C. vulgaris*) e macroalgas (*G. gracilis* e *U. rigida*) individuais, como também a sua mistura, apresentam atividade antibacteriana *in vitro* contra várias bactérias patogénicas, com resultados particularmente promissores contra *T. maritimum*. Além disso, a mistura das quatro algas foi identificada como uma fonte equilibrada e rica em nutrientes, nomeadamente proteína, lípidos, ácidos gordos, aminoácidos essenciais e hidratos de carbono. Para perceber o impacto destas algas na microbiota intestinal, foi testada uma inclusão de 8% das microalgas e macroalgas individuais (*N. oceanica* e *G. gracilis*) ou uma mistura destas

algas (4% de cada), quando incluídas em rações para robalo Europeu. Verificou-se que, enquanto a inclusão das algas individuais resultou numa diminuição da diversidade da comunidade microbiana presente no intestino posterior do robalo, este efeito redutor da diversidade foi mitigado pela inclusão destas algas numa mistura. Além disso, as algas testadas promoveram simultaneamente a proliferação de bactérias com potencial probiótico e uma diminuição da abundância de bactérias potencialmente prejudiciais.

O impacto na microbiota intestinal de uma mistura comercial de microalgas e macroalgas, constituída por *N. oceanica*, *C. vulgaris*, *G. gracilis* e *U. rigida*, em três níveis de inclusão (2%, 4% e 6%), foi também testado. Os resultados indicaram que a mistura de algas não afetou a diversidade microbiana no intestino posterior do robalo, mas levou a alterações nas proporções de grupos específicos de bactérias. No entanto, estes efeitos foram observados apenas no grupo com 2% de inclusão em comparação com o controlo e os outros níveis de incorporação, sendo que estas alterações parecem não estar interligadas com alterações da estrutura intestinal, crescimento ou resposta imune. No que diz respeito à histomorfologia intestinal, a mistura de algas, especialmente nos níveis de incorporação mais altos (4% e 6%), levou a um aumento da espessura da camada muscular, da altura das vilosidades, da largura da submucosa e da contagem de células caliciformes ácidas em comparação com o grupo controlo sem suplementação de algas. Quanto ao desempenho dos peixes, a inclusão da mistura de algas nas dietas de robalo promoveu o crescimento e a resposta imune nas mucosas após infeção com *T. maritimum*. De um modo geral, a mistura de algas, especialmente o nível de inclusão intermédio (4%), resultou numa resposta imune mais pronunciada facilitando a migração de monócitos e linfócitos para a pele e intestino. Esta resposta foi acompanhada por uma regulação positiva da expressão de genes associados à migração celular, proliferação e respostas pró-inflamatórias nestes tecidos.

Na fase final do projeto de doutoramento, foi padronizada uma metodologia para caracterizar os diferentes segmentos do trato gastrointestinal do robalo Europeu. Este conhecimento é crucial para avaliar e identificar formulações dietéticas capazes de modular a absorção de nutrientes, o estado imunológico e o crescimento de peixes de aquacultura. Foram também desenvolvidos protocolos para o isolamento de células imunes do rim e intestino do robalo Europeu, que servem como ponto de partida para o desenvolvimento de novas ferramentas *in vitro* com potencial para reduzir a dependência de experiências *in vivo*, abordando preocupações éticas e reduzindo a pegada ambiental da investigação em aquacultura.

Em conclusão, este projeto de doutoramento demonstrou o potencial promissor das microalgas e macroalgas para aumentar a robustez dos peixes, especialmente quando incluídas numa mistura. Especificamente, a mistura de algas melhorou o crescimento dos

peixes, tendo simultaneamente modulado eficazmente a saúde intestinal e a resposta imune do hospedeiro em resposta a uma infecção com o agente patogénico *T. maritimum*. De uma forma holística, os resultados obtidos destacam os benefícios da inclusão de misturas de algas em dietas de peixes com impacto positivo na saúde, performance e resistência a doenças. Este conhecimento poderá contribuir para a transformação da indústria da aquacultura no sentido de recorrer a práticas ambientalmente mais sustentáveis e éticas.

Abstract

As the global population continues to grow, the aquaculture sector is faced with the challenge of meeting the increasing demand for fish. Intensive fish farming often results in disease outbreaks, leading to high mortality rates and economic losses. Discovering innovative approaches to improve fish robustness and welfare is becoming increasingly essential, particularly due to challenges posed by rising antimicrobial resistance and its impact on aquaculture profitability. In this context, fish researchers have started exploring the potential benefits of including algae in aquafeeds to find natural resource-based solutions to enhance fish health, reduce reliance on antibiotics, and meet the demand for healthier food options.

At the initial phase of this PhD project, a comprehensive analysis was undertaken to unveil the specific potential of four commercially available microalgae (*Nannochloropsis oceanica* and *Chlorella vulgaris*) and macroalgae (*Gracilaria gracilis* and *Ulva rigida*) species. In addition, a combination of these algae was examined to uncover the collective potential of these biomasses. After determining their chemical and functional properties, the effects of the selected algae, when incorporated either individually or in combination in diets, on the intestinal microbiota and structure of European seabass (*Dicentrarchus labrax*) were investigated. Furthermore, a feeding trial utilizing algae-based feeds was conducted to gain further insights into the potential of these algae in enhancing the fish's resilience during a disease event. For this objective, European seabass were exposed to the Gram-negative marine bacterium, *Tenacibaculum maritimum*. The efficacy of the algae-based feeds was assessed by evaluating mortality rates and examining both the systemic and mucosal immune responses. This involved the analyses of the haematological profile, immune parameters in plasma, and gene expression patterns in the skin, intestine, and head-kidney tissues.

Antibacterial activities *in vitro* against several pathogenic bacteria were observed in both the individual microalgae (*N. oceanica* and *C. vulgaris*), macroalgae (*G. gracilis* and *U. rigida*), and their blend, with particularly promising results being seen against *T. maritimum*. Furthermore, the blend combination was noted as a well-balanced and nutrient-rich source of proteins, lipids, fatty acids, essential amino acids, and carbohydrates. Concerning the intestinal microbiota, the inclusion of individual micro- and macroalgae (*N. oceanica* and *G. gracilis*) at 8% of feed resulted in a decrease in the diversity of the posterior intestine microbial community in European seabass. However, this diversity-reducing effect on the intestinal bacterial communities was not detected when these algae were included in a blend (4% of each algae). Moreover, the inclusion of algae in European seabass feeds

exhibited a positive influence on the proliferation of bacteria with probiotic potential, while simultaneously decreasing the abundance of potentially harmful bacteria.

The incorporation of a commercial micro- and macroalgae blend, consisting of *N. oceanica*, *C. vulgaris*, *G. gracilis*, and *U. rigida*, at three inclusion levels (2%, 4%, and 6%), resulted in no changes in microbial diversity but affected specific groups of bacteria. However, these effects were observed only in the 2% inclusion group compared to both the control and the other incorporation levels. Nevertheless, these alterations did not correspond with changes in intestinal structure, growth, or immune responses. Notably, the algae blend, particularly at the highest incorporation levels (4% and 6%), impacted the intestinal histomorphology by increasing muscularis thickness, villi length, submucosa width, and the count of acid goblet cells compared to the control group without algae supplementation. In terms of fish performance, the inclusion of the algae blend in European seabass diets resulted in improved growth and an appropriate mucosal immune response when infected with *T. maritimum*. Notably, especially at the 4% inclusion level, the blend stimulated a more pronounced immune response by facilitating the migration of monocytes and lymphocytes to mucosal tissues. This was accompanied by an upregulation of genes associated with cell migration, proliferation, and pro-inflammatory responses in both skin and gut tissues.

In the final phase of the PhD project, a methodology was standardized for characterizing the different segments of the gastrointestinal tract of European seabass. This knowledge is crucial for evaluating and identifying the best dietary formulations able to modulate nutrient absorption, immunonutrition, and growth of farmed fish. Finally, protocols for the isolation of immune cells from the head-kidney and intestine of European seabass were developed. These protocols serve as a stepping stone for the development of novel *in vitro* tools that have the potential to reduce the reliance on *in vivo* experiments, addressing ethical concerns and reducing the environmental footprint of aquaculture research.

In conclusion, the incorporation of a blend of micro- and macroalgae in aquafeeds holds promising potential for boosting fish robustness. The blend not only promotes fish growth but also effectively modulates the intestinal health and the immune responses in European seabass when exposed to *T. maritimum*. These findings highlight the benefits of including algae blends in fish diets for overall fish performance and well-being. Overall, the results provided by this thesis might contribute to reshaping the aquaculture industry towards more sustainable and ethical practices.

Thesis Structure

This thesis is organized into eight chapters. The first chapter provides an overview of the state-of-the-art concerning the use of algae in fish diets. It begins by discussing the current challenges faced by aquaculture and how micro- and macroalgae, being rich sources of bioactive compounds, can help address these challenges. The chapter then presents a literature review on the modulation of fish health, specifically focusing on intestinal microbiota, histomorphology, immunity, and disease resistance using algae feeds. Given the high interest in the topic of nutritional immunomodulation within the sector and considering the 3Rs concept (Replacement, Reduction, Refinement), the introduction section also includes information about alternative models to evaluate the impact of functional diets on fish health. The first chapter concludes by providing an overview of the thesis objectives.

Chapters two to seven comprise the practical work of this PhD project and are presented in the form of manuscripts, following a paper-based structure, which resulted from this research project:

Chapter 2. Nutritional value, antimicrobial and antioxidant activities of micro- and macroalgae, single or blended, unravel their potential use for aquafeeds

Chapter 3. *Gracilaria gracilis* and *Nannochloropsis oceanica*, singly or in combination, in diets alter the intestinal microbiota of European seabass (*Dicentrarchus labrax*)

Chapter 4. Inclusion of algae blend in European seabass diets: Impacts on intestinal histomorphometry and microbiota

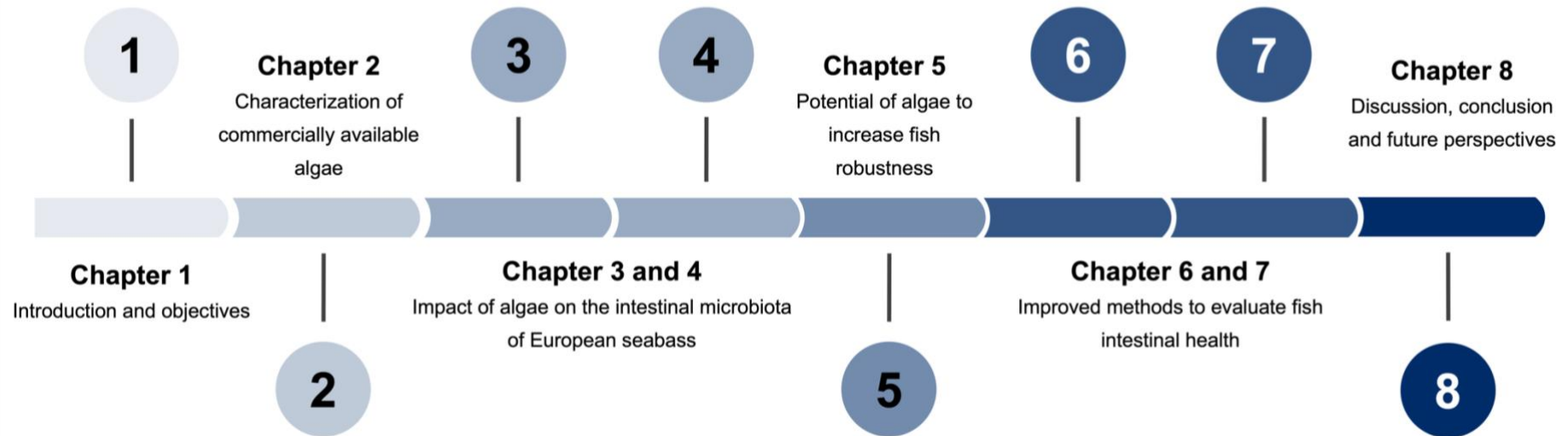
Chapter 5. Micro- and macroalgae blend modulates the mucosal and systemic immune responses of European seabass (*Dicentrarchus labrax*) upon infection with *Tenacibaculum maritimum*

Chapter 6. An in-depth characterisation of European seabass intestinal segments for assessing the impact of an algae-based functional diet on intestinal health

Chapter 7. Optimised protocols for isolation of immune cells from haematopoietic and mucosal organs of European seabass

The final chapter of the thesis offers a comprehensive discussion and presents the main conclusions drawn from this PhD research. Additionally, it outlines the bottlenecks, future approaches and potential avenues for further investigation based on the results obtained throughout this research project.

Thesis Structure Overview



Chapter 1.

General Introduction

1.1 Aquaculture: challenges and trends

The significance of aquaculture as a vital food-supply sector has steadily increased in recent years. This importance can be attributed to the demands of the global population for fish and other seafood products (Boyd et al., 2022). In 2021, over 157 million tonnes (89%) of the total production of aquatic animals were designated for human consumption. Notably, the aquaculture sector contributed 56% of the aquatic animals intended for human consumption, producing 90.9 million tonnes of farmed species (FAO, 2022). Fishes are a major source of macronutrients such as protein (constituting 17% of the total animal protein utilized in the food industry) and lipids (n-3 long-chain polyunsaturated fatty acids, n-3 LC-PUFA), as well as essential micronutrients, including vitamins (such as D, A, and B12), and minerals (like iodine, iron, and selenium) (EFSA, 2014; FAO, 2020).

The role of fish in providing these vital nutrients has necessitated a sustainable growth of the aquaculture sector. However, such a growth has been compromised by the limited and unstable supply of environmentally sustainable and cost-effective ingredients (Colombo et al., 2023; Tacon & Metian, 2015). Furthermore, heavy reliance on fish meal (FM) and fish oil (FO) that are golden sources of high-quality protein and n-3 LC-PUFAs puts substantial pressure on wild fish stocks. Hence, the aquafeed industry currently utilizes fish by-products obtained from both aquaculture and capture fisheries to fulfil its nutrient needs in feed formulations, aligning with the bio-circular concept (Sandström et al., 2022). Nevertheless, due to the inherent instability of the ingredients' market, the aquafeed industry is continually exploring alternative ingredients. Over the years, several potential substitutes for marine feedstuffs have emerged, including vegetable sources, algae, animal by-products and insects (Glencross et al., 2023).

At present, plant-based products (PP), which are labelled as the second-generation aquafeed ingredients, are the preferred alternatives (Colombo et al., 2023). However, the availability, cost, and sustainability of PP are constrained by current global socio-economic factors such as war, competition with the food and other animal feed sectors, and concerns about deforestation (Ben Hassen & El Bilali, 2022; Oyinlola et al., 2022). These limitations highlight the necessity for better alternatives. Partially replacing FM and FO with PP in aquafeeds for marine fish is feasible for several species, with modern commercial formulations already containing moderate to low levels of marine-origin resources. However, this replacement often comes with its set of challenges, including dietary imbalances, anti-nutritional elements, and potential deficiencies that may impact fish growth performance and overall health. This issue is particularly significant for carnivorous fish species, such as European seabass (*Dicentrarchus labrax*) (Colombo et al., 2023; Hua et al., 2019).

European seabass, primarily produced in the Euro-Mediterranean region, is an intensively reared marine species with high economic value. In 2021, European seabass production exceeded 250 tonnes, with Turkey (56%) and Greece (25%) accounting for the majority of the production (FEAP, 2023). Combined effect of industry demand-driven intensive farming and anthropogenic climate change can have a huge impact on the stress levels in fish and consequently weaken their immune system, rendering farmed aquatic animals more vulnerable to infections caused by pathogenic microorganisms (Boyd et al., 2020; Cascarano et al., 2021). European seabass production is frequently affected by disease outbreaks caused by Gram-negative bacteria (e.g., *Vibrio anguillarum*, *Photobacterium damsela* subsp. *piscicida* – Phdp, and *Tenacibaculum maritimum*), resulting in high mortality rates and significant economic losses (Cascarano et al., 2021). The aquaculture sector often resorts to antibiotics and chemotherapeutants to control bacterial outbreaks, a practice that not only fosters the emergence of drug-resistant pathogens but is also negatively perceived by consumers (Reverter et al., 2020). In this context, innovative solutions to enhance fish immunity and alleviate the burden of disease outbreaks are essential to address one of the most pressing public health concerns today, antimicrobial resistance, aligning with the One Health concept (Figure 1).

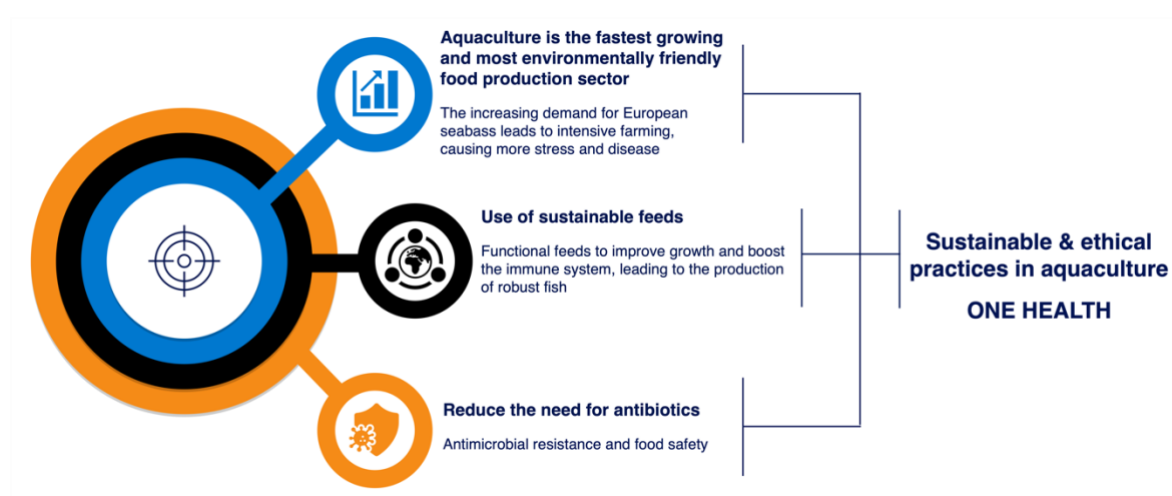


Figure 1. Schematic representation of the current challenges and needs of the aquaculture sector.

1.1.1 Functional diets in aquaculture: market and opportunities

The anticipated rise in both world population and subsequent fish consumption creates a strong likelihood for the expansion of aquaculture feed market. In fact, the market reached a volume of 47.2 million tons in 2022 and is projected to grow at a compound annual growth rate (CAGR) of 5.3%. Based on this growth pattern, Expert Market Research (2023) has provided a projected total volume of 64.2 million tons by 2028 (Figure 2). Aquafeeds constitute approximately 4.2% of the global compound feed tonnage, which amounted to

1.266 billion metric tons in 2022 (Alltech, 2023). As the aquaculture sector continues to grow, the importance of finding sustainable solutions for aquafeeds becomes more critical. The overall performance and disease resistance of fish is heavily depend on production conditions and proper nutrition, and efforts have been made to produce highly efficient diets supplemented with value-added products (Glencross et al., 2023).

In this context, there is a rising interest in additives and functional feeds since nutritional approaches offer promising solutions to alleviate diseases and enhance farmed fish's overall performance. According to MarketsandMarkets (2023), the global feed additives market is expected to follow a similar growth trend to that observed in the aquafeed market, with a CAGR of 5.8% from 2023 to 2028 (Figure 2). Functional aquafeeds have been successfully applied in aquaculture to improve fish growth performance, enhance digestibility, counteract the negative effects of antinutrients commonly found in PP-rich formulations, modulate the immune response, and increase resistance to diseases (Encarnação, 2016; Olmos Soto et al., 2015).

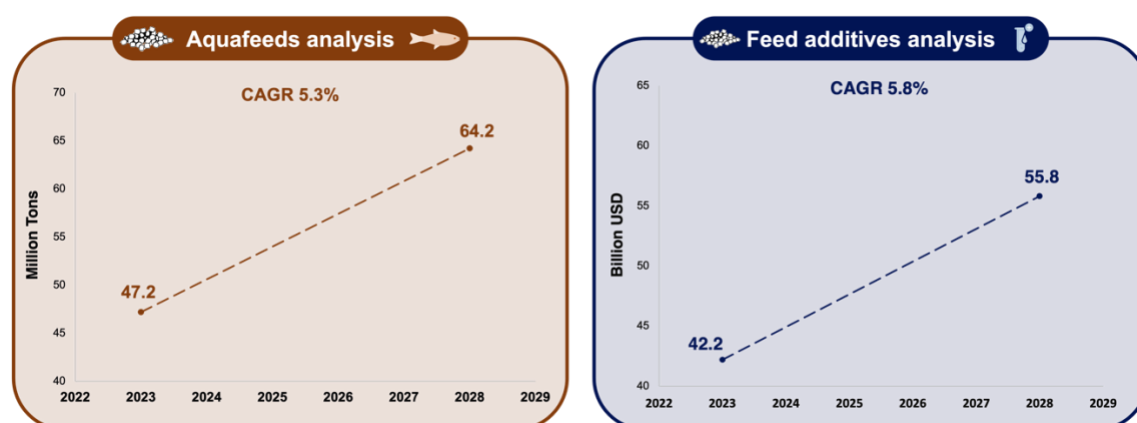


Figure 2. Growth expectations for the aquafeed and feed additive markets (2023-2028). Data retrieved from Expert Market Research (2023) and MarketsandMarkets (2023).

The significance of these functional feeds is particularly relevant today, given the tightening of legislation and policies concerning antibiotic use in food production, especially in the European Union (Walsh, 2022). In the case of European seabass, novel functional feeds have primarily been explored for their protective effects against *Vibrio* spp. and Phdp infections (Gisbert et al., 2018; Peixoto et al., 2019; Torrecillas et al., 2012). Additionally, vaccines that provide protection against *V. anguillarum* and Phdp infection in European seabass are currently available in the market (Miccoli et al., 2019). Nonetheless, functional feeds remain particularly relevant in aquaculture as they offer an important alternative, given the variable efficiency and constant need for updates associated with vaccines, which face

challenges exacerbated by environmental changes like climate change (Boyd et al., 2020; Mugwanya et al., 2022).

Regarding *T. maritimum*, the causative agent of tenacibaculosis, the only commercially available vaccine is specifically targeted for turbot (*Scophthalmus maximus*), which leads to a heavy reliance on antibiotics compared to other fish species. This pathogen affects not only seabass production but also many other commercial marine fish species such as Senegalese sole (*Solea senegalensis*), Atlantic salmon (*Salmo salar*) and gilthead seabream (*Sparus aurata*). The pathogenesis of tenacibaculosis is known to begin with the attachment of the pathogen to the external skin and mucus, followed by systemic infection (Avendaño-Herrera, Toranzo, & Magariños, 2006). However, the available reports on tenacibaculosis in farmed fish focus mainly on characterizing the mucosal and systemic immune responses, without addressing the efficacy of nutritional modulation in conferring protection against *T. maritimum*. Despite the efforts to comprehend immune responses during the infection, there are still gaps in the understanding of pathogenesis of this disease. Hence, further studies should be directed towards elucidating the key frontline immune mechanisms that different fish species employ to combat this disease (Escribano et al., 2020; Ferreira et al., 2023a; Guardiola et al., 2019).

Given the growth of the aquaculture sector and the functional feed market, it is now more relevant than ever before to target and develop new additives. This emphasis on additives is crucial for aquaculture to not only to promote fish growth and health but also to align with the 3 pillars of sustainability (Eroldoğan et al., 2023). Indeed, the search for novel natural feed additives and ingredients for aquaculture is intensifying, with algae taking the forefront as a prominent focus due to their diverse properties and potential benefits. Micro- and macroalgae, can be incorporated into aquafeeds to improve fish immune and antioxidant status, enhance disease resistance, and promote overall health (Ferreira et al., 2020; Peixoto et al., 2019; Peixoto et al., 2016; Qiao et al., 2019).

1.2 Algae as rich sources of bioactive components for aquafeeds

Algae produce a wide variety of metabolites, such as pigments (e.g. carotenoids), polyphenols, polysaccharides, sterols, vitamins and fatty acids with potential antioxidant and antibacterial activities (Abu-Ghannam & Rajauria, 2013; Sansone & Brunet, 2020). These bioactive compounds are particularly of interest to the feed industry because of their ability to impart positive effects that can have a bearing on the health of farmed animals. Compounds with antioxidant properties are those that can counteract the oxidative damage by direct neutralization of free radicals, by interfering with the expression or activity of free radical-generating enzymes or by increasing the expression of the endogenous antioxidant

enzymes (Lü et al., 2010). On the other hand, these compounds exert their antimicrobial action through two possible mechanisms: bactericidal activity, by direct destruction of microorganisms (e.g. alter bacteria cell wall permeability, thereby causing the loss of cellular integrity); or bacteriostatic activity, by inhibiting the growth of microorganisms (Abu-Ghannam & Rajauria, 2013). Although most nutritional studies in fish have focused on the potential of a single alga species to reveal their suitability in aquafeeds, simultaneous inclusion of two or more micro- and macroalgae may allow synergistic antibacterial and antioxidant activities of distinct species. Functional diets can be formulated by including the bioactive compounds and/or extracts from algae or by using whole algae without prior processing. Among these alternatives, the feed industry generally prefers the latter due to its competitive prices.

1.2.1 Microalgae

Microalgae are a diverse group of unicellular or simple multicellular photosynthetic and microscopic algae. There are also heterotrophic and mixotrophic microalgae. Autotrophic microalgae are essential components of the earth's ecosystem, both aquatic (salt and fresh water) and terrestrial systems. Some species have been adapted to grow even in harsh conditions. Microalgae may be produced in open shallow ponds or closed photobioreactors, with open culture systems being preferred for large-scale production (Khan et al., 2018). Several commercial applications of microalgae have been reported, including their utilization to provide essential nutrients for both humans and animals, as well as their use in cosmetics, fertilizers, and biodiesel production (Mata et al., 2010; Spolaore et al., 2006; Valente et al., 2020).

The rising interest to include microalgae in fish feeds can be attributed to the content of high-quality protein (40-70%), lipids including n-3 PUFAs and bioactive compounds (Shah et al., 2018). In the past years, several efforts have been made to understand their potential as ingredients to replace both FM and FO or as feed supplements (Valente et al., 2021). The inclusion of microalgae in aquafeeds has been previously associated with additional health benefits that included stimulation of immune response and improved antioxidant status (Cerezuela et al., 2012b; El-Habashi et al., 2019; Messina et al., 2019; Teimouri et al., 2019).

As the market for functional food increases, a rise in demand for microalgae and their derivatives has also been observed, with about 30% of microalgal production being currently directed for animal feed applications (Hamidi et al., 2019). In this context, *Nannochloropsis oceanica* and *Chlorella vulgaris* are two microalgae of relevance to aquaculture. *Nannochloropsis* sp. are small sized green algae belonging to the class Eustigmatophyceae

and specifically known for both their high-quality protein and fatty acids, namely eicosapentaenoic acid (EPA) (Hemaiswarya et al., 2011). *Nannochloropsis* sp. are also valuable sources of carotenoids (i.e. zeaxanthin, canthaxanthin and astaxanthin), carbohydrates and other compounds with possible immunostimulatory effects crucial for enhancing fish health (Cerezuela et al., 2012b; Shah et al., 2018). *Chlorella* sp. are unicellular photosynthetic microalgae usually found in freshwater environments. *Chlorella* is one of the most industrially produced genera of microalgae, with a global production of 5000 tons per year. *Chlorella* sp. have also a wide range of bioactive compounds, namely carotenoids, β -1,3-glucan, vitamins, and polyphenols that possess antioxidant and antimicrobial activities (García et al., 2017; Hamidi et al., 2019). Hence, several studies have been conducted to understand the potential of this genus to be used as partial FM and FO replacers (Haas et al., 2016; Qiao et al., 2019; Valente et al., 2019).

1.2.2 Macroalgae

Macroalgae, commonly known as seaweeds, are multicellular and macroscopic algae that encompass a diverse range of species grouped into three main categories on the basis of their pigmentation: Rhodophyta (red), with phycoerythrin and phycocyanin as the main pigments and agar and carrageenan as the primary polysaccharides; Chlorophyta (green), mainly composed by the chlorophyll a and b (pigments) and the polysaccharide ulvan; and Phaeophyta (brown), rich in fucoxanthin pigment and several polysaccharides, namely alginates, laminarins, fucans and cellulose. Seaweeds are found in different environments, including intertidal and subtidal coastal zones and estuarine habitats, and are able to sustain harsh conditions (O'Sullivan et al., 2010; Wan et al., 2019). The majority of seaweeds are cultivated for various commercial applications; for example, the entire biomass or specific compounds are incorporated into human diets and supplements, animal feeds, fertilizers and they are used as feedstock for biofuel production (Ghadiryanfar et al., 2016; Tiwari & Troy, 2015).

Seaweeds are natural sources of bioactive compounds, such as pigments and polysaccharides, which hold significant value for the food and feed industries. Studies have been conducted to understand the potential applicability of the whole biomass of seaweeds and their specific compounds in aquafeeds. Moreover, some macroalgae are already produced as part of integrated multitrophic aquaculture (IMTA) systems, taking advantage of the nutrients produced by the fish farms. This approach enables the recycling of nutrients, thereby enhancing water quality and mitigating the environmental footprint (Abreu et al., 2011; Stévant et al., 2017). The *in vitro* antimicrobial and antioxidant activities of extracts from representative species of Chlorophyta, Rhodophyta and Phaeophyta have been

previously demonstrated (Bansemir et al., 2006; Chew et al., 2008; Narasimhan et al., 2013). *In vivo* nutritional studies have been performed on relevant farmed fish species, such as European seabass, gilthead seabream and rainbow trout (*Oncorhynchus mykiss*), to unravel the impact of seaweed supplementation. In fact, beneficial effects were reported in terms of immune and antioxidant status as well as resistance to stress and bacterial diseases (Araújo et al., 2016; Magnoni et al., 2017; Peixoto et al., 2019).

Among seaweeds, red and green algae, such as *Gracilaria gracilis* and *Ulva rigida*, have spurred the interest of the fish feed sector. Species belonging to the genera *Gracilaria* and *Ulva* are economically valuable resources due to their capacity to achieve high biomass yields and their content of biologically active phytochemicals (Bruhn et al., 2011; Capo et al., 1999; Francavilla et al., 2013). *G. gracilis* and *U. rigida* are edible seaweeds that have been traditionally used as food, but there is untapped potential in the extraction of their polysaccharides. Nevertheless, agar from *G. gracilis* is already used by food, pharmaceutical and biotechnology industries (Torres et al., 2019). On the other hand, the polysaccharide ulvan, a cell wall component of different green macroalgae, including *U. rigida*, remains underexplored despite the broad range of biological activities displayed by this compound (e.g. immunostimulant, antioxidant, anti-viral, anticancer and anticoagulant) (Kidgell et al., 2019). Some species of *Gracilaria*, namely *G. gracilis*, are also rich in arachidonic acid (ARA), with values ranging from 16 to 48% total fatty acids, depending on the season (Francavilla et al., 2013). ARA is an essential LC-PUFA in marine fish nutrition due to its role as an eicosanoid precursor, which is involved in several physiological processes, including immunity and stress response (Tocher, 2003; Torrecillas et al., 2017b). Thus, seaweeds serve as multifaceted resources that can offer an array of valuable products for enhancing the value of aquafeeds.

1.3 Modulation of fish health using diets with algae inclusion

The intricate relationship between fish, their intestinal microbiota, histomorphology, and immune system is a central focus of research in the field of aquaculture and fish health. The intestine of fish hosts a dynamic community of microorganisms that are constantly interacting with the host due to their evolutionarily determined intricate relationship (Egerton et al., 2018). Simultaneously, the morphology and functionality of the fish intestine play a crucial role in nutrient absorption. By maintaining its normal structure, the intestine ensures proper responses elicited by the components of its physical and chemical barrier (Dawood, 2021). In addition, fish possess a well-defined immune system that encompasses both innate and adaptive components, working together to defend the host against diseases (Rauta et al., 2012). The interplay among members of the triad – microbiota, intestinal

structural and immune components – is acknowledged as crucial in determining the overall health and disease resistance of farmed fish.

In recent years, a promising avenue of research has emerged, with a shift in focus towards exploring the ability of micro- and macroalgae to modulate these essential health parameters. Algae have gained attention for their capacity to influence the composition of the fish gut microbiota (Gonçalves et al., 2022; Li et al., 2023), promote intestinal morphological alterations (Liu et al., 2022a; Passos et al., 2021), and boost the immune system (Cerezuela et al., 2012b; Peixoto et al., 2019). For a comprehensive overview of the effects of various micro- and macroalgae on growth performance, intestinal morphology, functionality, microbiota, and immune responses in different fish species, please refer to Table 1 at the end of section 1.3. This multidimensional approach that considers the triad offers a holistic perspective on how algae can contribute to improving fish health and welfare in aquaculture, besides enhancing the sustainability of the industry.

1.3.1 Fish intestinal microbiota

The gastro-intestinal (GI) tract of vertebrates hosts diverse and dynamic microorganisms collectively referred to as the microbiota. In both mammals and fish, microbe–microbe and host–microbe interactions, which determine the complexity of the intricate assemblage of gut microbes, are known to play a crucial role in the maintenance of key physiological functions that ultimately affect host health (Cerf-Bensussan & Gaboriau-Routhiau, 2010; Egerton et al., 2018). In vertebrates, the bidirectional dependence of the gut microbiota and immune capacity are well described, and a balanced microbiota-immune system interaction is essential for various aspects such as resistance to invasive pathogens that decides the host health (Belkaid & Hand, 2014). The endogenous microbiota is also known to have implications on fish immune system and health status (Gómez & Balcázar, 2008). By following the paths taken by human and mammalian research, aquaculture scientists have targeted the GI tract of fish to study the associated microbiota. Although most studies focused on the nutritional manipulation of farmed fish intestinal microbiota to improve welfare and nutrition, several other factors can also be major drivers in modulating these endogenous populations, including seasonal variations, stress and water quality (Ringø et al., 2016). However, the lack of in-depth knowledge of the functional role that specific microbial populations play on fish health is still one of the main obstacles for an accurate interpretation of the results from microbiota studies (Egerton et al., 2018).

The analysis of gut microbiota includes various criteria, such as alpha and beta diversity assessments, along with taxonomic profiling. Alpha diversity quantifies the species richness (number of different species) and species evenness (relative abundance of each species)

within a specific sample, representing the diversity within a particular community. In contrast, beta diversity examines differences in species composition or structure between distinct samples (Kers & Saccenti, 2022). Researchers have explored the dietary modulation of fish gut microbiota either by administering probiotics and prebiotics or by modifying the composition of dietary proteins and lipids (Egerton et al., 2018). Recent studies have also reported the impact of novel diets including added-value products, such as algae that provide benefits beyond basic nutrition. These studies are either focused on the impact of replacing FM or FO or feed supplement with algae. The inclusion of 5% dietary *Schizochytrium limacinum* as partial FO substitute significantly increased the intestinal bacterial diversity in rainbow trout without changing the overall microbial community structure. Nevertheless, there was an over representation of lactic acid bacteria (LAB), which are commonly considered beneficial (Lyons et al., 2017). Short-term feeding of gilthead seabream with diets supplemented with the microalga *N. gaditana* at 7.5 g kg⁻¹ increased the gut microbiota richness, but not significant when compared with fish fed the control diet. These dietary regimes did not affect the mucosa integrity and digestive capacity (Jorge et al., 2019). On the contrary, Cerezuela et al. (2012a) reported a decreased bacterial diversity, accompanied by oedema and inflammation signs, in the anterior intestine of gilthead seabream fed diets with either *Tetraselmis chuii* or *Phaeodactylum tricornutum* (100 g kg⁻¹).

Concerning seaweeds, when FM was partially replaced by the macroalgae *G. cornea* or *U. rigida* there was an associated increase in gut microbial diversity in gilthead seabream fed 15% of one of the seaweeds. However, with the increase in inclusion level (25%) of *U. rigida* the diversity was decreased significantly. Interestingly, this treatment (25% inclusion of *U. rigida*) was also associated with a significantly higher final body weight (Rico et al., 2016). Although a decreased bacterial diversity may not always be detrimental to the host, a reduction in the intestinal microbial diversity and unfavourable shift in the bacterial composition have been linked to diseases in fish (Li et al., 2016). A recent study on European seabass fed diets supplemented with either 2.5 or 5% of whole biomass of *G. gracilis* or 0.4% of the algal extract revealed the changes in the composition of intestinal bacterial communities. Notably, the diets did not affect the overall diversity of the microbiota. Nonetheless, the algae diets led to a reduction in the abundance of certain bacterial genera, including *Photobacterium*, *Dermacoccus*, *Staphylococcus* and *Sphingomonas*. Moreover, the study predicted changes in functional pathways related to metabolism and the synthesis of protective compounds, such as ectoine, as well as alterations in succinate and pyruvate fermentation (Gonçalves et al., 2022).

There are also studies that have investigated the influence of particular compounds derived from macroalgae on the composition of the intestinal microbiota (Ashouri et al., 2020; Gupta

et al., 2019). Alginate oligosaccharide that is derived from *Laminaria* sp. is a candidate prebiotic, and its inclusion at 0.5% in the diets for Atlantic salmon caused a shift in the microbiota profile of the distal intestine. This shift stimulated the growth of certain bacteria belonging to Proteobacteria, Spirochaetes and Actinobacteria, some of which have the potential to degrade carbohydrates and produce butyrate (*Aliivibrio logei*, *Aquabacterium parvum*, *Brevinema andersonii* and *Achromobacter insolitus*). A higher supplementation level (2.5%) resulted, however, in a reduction of the microbial diversity, which led the authors to conclude that low inclusion of macroalgae-derived alginate oligosaccharide can induce some prebiotic effects, highlighting the potential of seaweed components to modulate the gut microbiota of farmed fish (Gupta et al., 2019). Nonetheless, the knowledge of the impact of dietary algae supplementation on fish microbiota is limited.

In summary, these findings suggest that dietary approaches using algae, whether as ingredients or supplements, can influence the composition of fish gut microbiota. However, it is important to note that the effects vary depending on the fish species, the specific type of algae used, and the inclusion levels. Most studies primarily focus on the effects of individual algae. An intriguing area for further exploration is the use of a combination of various micro- and macroalgae in aquafeeds, rather than relying on a single algal source. This approach may lead to synergistic effects on gut microbiota modulation, yet it remains an understudied field.

1.3.2 Fish intestinal histomorphology

The intestine has a crucial role in shaping the health of farmed fish. This organ not only performs the crucial function of nutrient absorption but also serves as a physical and chemical barrier against invading pathogens (Dawood, 2021). The emerging feed ingredients that are expected to have a health transformative potential can mould the digestive tract features. Valuable insights into the effects of novel feed ingredients on the morphology and functions of the fish intestine can be obtained through integrated approaches that combine histomorphometric indices, and molecular and biochemical biomarkers (Tan et al., 2020; Xie et al., 2021a). Currently, the assessment of fish intestinal health relies on classical histological parameters, such as villi shortening, with many studies reporting these morphological changes using semi-qualitative analyses (Basto-Silva et al., 2022; Iqbal et al., 2022). An increasing shift towards quantitative approaches can enhance our understanding of fish intestinal health. Such a strategy to move beyond semi-qualitative analyses can provide more robust insights into this critical aspect of fish health (Batista et al., 2020a; Li et al., 2023; Liu et al., 2022a; Passos et al., 2021; Torrecillas et al., 2019).

Indeed, several studies have assessed the impact of diets supplemented with micro- and macroalgae on fish performance. Intestinal histomorphological assessment, in these studies, has provided valuable insights into how these products can influence the overall health of fish. For instance, the microalga *Scenedesmus almeriensis* in the diets for gilthead seabream can enhance the absorptive capacity of the intestinal mucosa, by increasing the length (i.e. the absorptive surfaces) of both villi and microvilli (Vizcaíno et al., 2014). Similarly, a diet with 36% *Chlorella sorokiniana* was found to increase the villi height and muscularis thickness and upregulate the expression of the tight junction protein genes (*cld1*, *ocln*, and *zo-1*) in the intestine of rainbow trout (Liu et al., 2022a). In contrast, a 74% inclusion of *C. vulgaris* to replace the total FM in the diet of largemouth bass (*Micropterus salmoides*) reduced the villus height and the activity of mucin 2 and some immune parameters in the intestine, as reported by Li et al. (2023).

Numerous studies have highlighted the positive effects of incorporating macroalgae-derived products in fish feeds. However, it is essential to note that, unlike microalgae, studies involving seaweeds typically focus on lower inclusion levels, ranging from 2% to 10%. Indeed, Passos et al. (2021) noted intestinal morphological alterations in European seabass fed diet with 2.5% *G. gracilis*. These changes involved broader villi that corresponds to improved absorptive area, and a higher count of acid goblet cells, which play a protective role against bacterial translocation. Likewise, *Ulva ohnoi* at a 5% inclusion level in the diet of Senegalese sole increased the villus height, enterocyte height, and the total absorptive surface of enterocytes, after a 45-day feeding trial. These morphological alterations were also marked by an increase in alkaline phosphatase activity, a well-known marker of intestinal integrity and nutrient absorption (Vizcaíno et al., 2019). Nonetheless, it is important to note that negative effects of macroalgae supplementation have also been documented. For instance, when *Ascophyllum nodosum* was included at a 10% level in diets for Atlantic cod (*Gadus morhua*), there was a reduction in villus height and widening of the lamina propria, possibly indicating the infiltration of inflammatory cells. Fish fed with the *A. nodosum*-supplemented diet also exhibited poorer growth performance. These findings led the authors to suggest that this macroalgae, at such high inclusion level, may not be ideal for Atlantic cod (Keating et al., 2021).

In conclusion, both micro- and macroalgae can have a positive impact on gut morphology and functionality in fish, but the specific results are highly dependent on various factors, including the species and inclusion level of algae as well as the fish species. To accurately evaluate the effects of novel feeds on intestinal morphology that reflects fish health, it is essential to meticulously select the intestinal section, clearly define the parameters to be evaluated, and specify the type of approach, quantitative or semi-quantitative, employed for the analysis. This level of detail is crucial for obtaining accurate and reliable results,

ultimately enhancing comparability across different studies (Vatsos, 2021). To draw meaningful conclusions in the ever-evolving field of fish intestinal health, careful consideration of these factors and comprehensive reporting of methodology is essential.

1.3.3 Fish immunity and disease resistance

The immune system in fish, much like in higher vertebrates, involves both cellular and humoral components, operating at two essential ways: non-specific responses under the innate defence system and specific acquired responses of the adaptive immune system. The head-kidney, and to a lesser extent, the spleen, play critical roles in fish immunity as haematopoietic organs. The head-kidney cells are involved in processes like phagocytosis, antigen processing, immunoglobulin (Ig) M formation, and immune memory acquisition (Rauta et al., 2012). Additionally, mucosal tissues, such as the skin, gills, nasopharynx, and gut, are intimately connected to these defence mechanisms. The skin, gills and intestine, along with their associated mucus, act as primary physical and chemical barriers. The gut mucosa, known as gut-associated lymphoid tissues (GALTs), is rich in immune cells, including intraepithelial lymphocytes (T cells located between epithelial cells) and lamina propria leucocytes (mainly lymphocytes, macrophages, granulocytes, and plasma cells). Together with the gut microbiota, GALTs play a crucial role in maintaining host mucosal homeostasis, affecting both fish nutrition and immune status (Parra et al., 2015; Rombout et al., 2011).

The innate defence system includes cellular elements like monocytes/macrophages, dendritic cells, granulocytes (e.g., neutrophils), and cytotoxic cells that are involved in phagocytosis and inflammatory responses. On the humoral level, mucosal tissues and plasma contain various non-specific immune factors such as antibacterial peptides, lysozyme, peroxidase, complement factors, and other lytic enzymes, serving as the first line of defence. The adaptive defence mechanism relies on immunological memory to generate responses through antibody-mediated immunity and cellular immune responses mediated by T-lymphocytes. Fish-specific immune components also involve B- and T-lymphocyte populations, T-cell receptors, and products of the major histocompatibility complex (Magnadóttir, 2006; Rauta et al., 2012; Stosik et al., 2021).

The immune responses and disease resistance in fish are influenced not only by genetic and environmental factors but also by nutrition. Most studies that assess how fish immunity is influenced by dietary factors focus on blood and plasma assays, examining parameters such as complement activity, lysozyme activity, total IgM, and total and differential leucocyte counts (Dawood, 2021). Nutritional modulation of fish immunity has been explored for the past 40 years, with a primary focus on specific nutrients like vitamins, fatty acids, amino

acids; and, more recently, prebiotics and additives such as glucans, mannan oligosaccharides, and probiotics (Gatlin & Yamamoto, 2022; Kiron, 2012). In recent years, there has been an increasing interest to understand the impact of aquafeeds supplemented with algae on fish immune responses and resistance to pathogenic bacteria. Most of these studies have concentrated on the effects of individual micro- and macroalgae or a mix of either microalgae or seaweeds.

For example, *Tetraselmis* sp. or *Chlorella* sp. incorporated in both FM- and soybean meal-based diets improved the survival of zebrafish larvae after infection with *Edwardsiella tarda* (Bravo-Tello et al., 2017). Similarly, diets supplemented with *C. vulgaris* improved both immune and antioxidant status of Nile tilapia (*Oreochromis niloticus*) exposed to the herbicide penoxsulam, and the fish also exhibited improved resistance to *Aeromonas sobria* infection. In this case, inclusion of the microalga led to an increased expression of immune-related genes (*il-1 β* and *tnf- α*), concomitant with an elevation of various humoral factors and improved antioxidant status (Galal et al., 2018). Cerezuela et al. (2012b) also observed some immunostimulatory effects of different microalgae (*N. gaditana* or *T. chuii* or *P. tricornutum*) incorporated in the diets of gilthead seabream; increased haemolytic complement activity, phagocytic ability and capacity, and up-regulation of some immune-related genes. Overall, *P. tricornutum* seemed to have the highest immunomodulatory effects amongst the tested microalgae. On the other hand, diets with defatted biomass of *Nannochloropsis* sp. as a partial substitute of FM (i.e. 5, 10 and 15%) did not alter the plasma lysozyme, alternative complement pathway and peroxidase activities in European seabass (Valente et al., 2019).

Similar to microalgae, seaweeds also are known to impart immunomodulatory effects on fish. For instance, adding 5% *Gracilaria* sp. aqueous extract in aquafeeds for European seabass elevated the lysozyme activity and affected immune-related pathways, as well as caused a delay in photobacteriosis when fish were infected with the pathogenic bacteria Phdp following a 80 days feeding trial (Peixoto et al., 2019). Fumanal et al. (2020) reported a modulation of the immune response in Senegalese sole fed diets with 5% *U. ohnoi* and subsequently infected with Phdp, with increased expression of genes encoding for complement, lysozyme, and *tnf- α* . The enhanced immune response was accompanied by a lower bacterial load in fish liver, although mortality rates remained similar. A study on the effects of dietary inclusion of *Laminaria digitata* and a commercial blend of seaweeds demonstrated a more efficient immune response in LPS-challenged Atlantic salmon consuming the seaweed-supplemented diets. The blend of macroalgae was associated with a higher number of differentially expressed immune pathways compared with the inclusion of single alga, indicating a synergistic enhancement of the required responses by several algae (Palstra et al., 2018).

In general, both microalgae and seaweeds have shown the potential to act as immunostimulants in fish. Further research is needed to understand the short- and long-term impacts of algae-supplemented diets on fish immunity in order to define the most adequate nutritional strategy to enhance fish robustness. To obtain reliable results, studies should consider immune responses at both local and systemic levels, and pathogen exposure experiments should be well-designed, accounting for bacterial strain, stress duration, and method of exposure (Kiron, 2012).

To sum up, further research in this field – modulation of fish health using algae-supplemented diets – has the potential to yield a deeper understanding of how algae species with inherent added-value, when included in diets for farmed fish, interact with intestinal microbial communities, influence intestinal histomorphology, and modulate the immune system. This knowledge can be instrumental in driving the development of innovative strategies and tools aimed at enhancing fish robustness, health, and overall welfare. This, in turn, can help reduce the reliance on antimicrobials while simultaneously enhancing production efficiency and the overall sustainability of the aquaculture sector.

Table 1. Summary of the effects of various micro- and macroalgae on growth performance, morphology of the intestine, its functionality and associated microbiota, immune/antioxidant responses, and disease resistance in different fish species.

Fish Species and Weight 	Algal inclusion in diet and Trial Duration 	Growth Performance 	Intestinal Morphology and Functionality 	Intestinal Microbiota Structure and Composition 	Immune Response and Disease Resistance 	Reference 
Asian sea bass (<i>Lates calcarifer</i>) 12 g	Macroalgae <i>Undaria pinnatifida</i> -derived sodium alginate inclusion at 5 and 10 g kg ⁻¹ feed 42 days	↔ FBW	↑ Villus height and apparent villus surface ↑ Muscularis thickness ↑ Total protease, ↑ Trypsin and lipase (for 10 g kg ⁻¹ diet)	↑ Lactic acid bacteria	↑ Antioxidant enzymes (CAT and GST) in liver ↑ Lysozyme in intestine	Ashouri et al. (2020)
Atlantic cod (<i>Gadus morhua</i>) 123 g	Macroalgae inclusion at 10% in feed (partially replacing FM and potato starch): 1) <i>Ascophyllum nodosum</i> 2) <i>Ulva rigida</i> 12 weeks	<i>A. nodosum</i> : ↓ FBW <i>U. rigida</i> : ↔ FBW	<i>A. nodosum</i> : ↑ Lamina propria width ↓ Villus height <i>U. rigida</i> : ↔ Gut morphology	<i>A. nodosum</i> : ↑ <i>Psychrosomonas</i> spp., <i>Propionigenium</i> , <i>Clostridium</i> sensu stricto, and <i>Rhodospirillaceae</i> ↓ Richness and diversity <i>U. rigida</i> : ↔ Diversity and community composition	Not evaluated	Keating et al. (2021)
Atlantic salmon (<i>Salmo salar</i>) 378 g	Macroalgae inclusion at 10% in feed (partially replacing vegetable products): 1) <i>Laminaria digitata</i> 2) Commercial blend of seaweeds 42 days feeding trial followed by LPS challenge	↔ FBW	Not evaluated	Not evaluated	↔ Haematocrit and haemoglobin Post-LPS challenge <i>L. digitata</i> : ↑ Expression of major histocompatibility complex class I-related gene protein ↓ Interferon-gamma-mediated signalling pathway Commercial blend of seaweeds: ↑ Differentially expressed pathways compared with the single alga ↑ Expression of genes related to receptor-mediated endocytosis, cell adhesion and lipopolysaccharide and inflammatory response	Palstra et al. (2018)

Fish Species and Weight 	Algal inclusion in diet and Trial Duration 	Growth Performance 	Intestinal Morphology and Functionality 	Intestinal Microbiota Structure and Composition 	Immune Response and Disease Resistance 	Reference 
Atlantic salmon 186 g	Macroalgae <i>Laminaria</i> sp.-derived alginate oligosaccharide inclusion at 0.5 and 2.5% in feed 9 weeks	Not evaluated	Not evaluated	↓ Diversity (2.5% only) ↑ <i>Aliivibrio logei</i> , <i>Aquabacterium parvum</i> , <i>Brevinema andersonii</i> and <i>Achromobacter insolitus</i> – bacteria with possible capacity to degrade carbohydrates and produce butyrate	Not evaluated	Gupta et al. (2019)
Atlantic salmon 5 g	Macroalgae <i>Asparagopsis taxiformis</i> inclusion in feed: 1) Whole algae at 3% 2) Methanolic extract at 0.6 and 1.2% 2 and 4 weeks	↑ FBW	Not evaluated	↔ Composition ↑ Diversity ↑ <i>Shewanella</i> and <i>Aeromonas</i> ↓ <i>Streptococcus</i> and <i>Pseudomonas</i>	↔ Lysozyme and ACH50 in plasma ↔ Respiratory burst, and phagocytic index ↓ Glucose (2 weeks)	Thépôt et al. (2022)
European seabass (<i>Dicentrarchus labrax</i>) 22 g	Microalgae <i>Nannochloropsis</i> sp. inclusion at 5, 10 and 15% in feed (partially replacing FM) 93 days	↔ FBW	↔ Gut morphology	Not evaluated	↔ Lysozyme, ACH50 and peroxidase in plasma	Valente et al. (2019)
European seabass 12 g	Macroalgae <i>Gracilaria</i> sp. aqueous extract inclusion at 5% in feed 80 days feeding trial followed by infection trial with <i>Photobacterium damselae</i> subsp. <i>piscicida</i> (Phdp)	↔ FBW	Not evaluated	Not evaluated	↑ Lysozyme in plasma ↑ Expression of <i>il34</i> , <i>ccr9</i> and <i>cd33</i> (inflammation) in head-kidney Delayed photobacteriosis (Phdp infection) ↓ Expression of <i>grp-78</i> , <i>grp-170</i> , <i>grp-94</i> and <i>grp-75</i> (stress-responsive heat shock proteins) in liver ↓ Expression of <i>mif</i> , <i>il-1β</i> , <i>defb</i> , <i>a2m</i> and <i>myd88</i> (cytokine signalling) in head-kidney	Peixoto et al. (2019)

Fish Species and Weight 	Algal inclusion in diet and Trial Duration 	Growth Performance 	Intestinal Morphology and Functionality 	Intestinal Microbiota Structure and Composition 	Immune Response and Disease Resistance 	Reference 
European seabass 19 g	<i>Arthrospira platensis</i> (formerly Spirulina) inclusion at 15% in feed (partially replacing vegetable products in a FM and FO free diet) 93 days	↓ FBW	Not evaluated	↔ Richness and diversity ↑ <i>Persicirhabdus</i> , <i>Methylobacterium</i> , <i>Acinetobacter</i> , and <i>Sediminibacterium</i> ↓ Proteobacteria	Not evaluated	Pérez-Pascual et al. (2020)
European seabass 30 g	Macro- and microalgae inclusion, single or in combination (partially replacing FM) in feed: 1) <i>Gracilaria gracilis</i> 8% 2) <i>Nannochloropsis oceanica</i> 8% 3) <i>G. gracilis</i> 4% + <i>N. oceanica</i> 4% (blend) 106 days	↔ FBW	↔ Gut morphology (villus length, width and muscularis thickness) ↑ Neutral goblet cells (<i>N. oceanica</i>) ↓ Brush border membrane enzyme activities (<i>N. oceanica</i>)	Not evaluated	↔ Total cholesterol, protein, albumin, lysozyme and peroxidase in plasma ↑ Glucose in plasma (<i>G. gracilis</i>) ↓ Triglycerides ↓ Antioxidant enzymes (total glutathione and GPx) in liver (blend) ↓ ACH50 in plasma (blend)	Batista et al. (2020a)
European seabass 17 g	Macroalgae <i>Gracilaria gracilis</i> inclusion in feed: 1) Whole algae at 2.5 and 5% 2) Extract at 0.4% 47 days	↔ FBW	↑ Muscularis outer longitudinal and inner circular layers (whole algae 2.5 and extract) ↑ Villus width (whole algae 2.5) ↑ Acid goblet cells ↓ Neutral goblet cells	Not evaluated	↔ Haematological parameters and peroxidase ↑ Lysozyme in plasma (whole algae 5) ↑ SOD activity in liver ↑ tGSH activity in liver (whole algae 2.5) ↓ Lipid peroxidation in liver (whole algae 2.5)	Passos et al. (2021)

Fish Species and Weight 	Algal inclusion in diet and Trial Duration 	Growth Performance 	Intestinal Morphology and Functionality 	Intestinal Microbiota Structure and Composition 	Immune Response and Disease Resistance 	Reference 
European seabass 17 g	Macroalgae <i>Gracilaria gracilis</i> inclusion in feed: 1) Whole algae at 2.5 and 5% 2) Extract at 0.4% 47 days	↔ FBW	Not evaluated	↔ Richness and diversity ↓ <i>Photobacterium</i> and <i>Dermacoccus</i> ↓ <i>Staphylococcus</i> and <i>Sphingomonas</i> (extract) ↑ Predicted functions related to metabolism and biosynthesis of protective compounds (e.g., ectoine) ↑ Predicted functions related to succinate and pyruvate fermentation	Not evaluated	Gonçalves et al. (2022)
Gilthead seabream (<i>Sparus aurata</i>) 50 g	Microalgae inclusion at 100 g kg ⁻¹ feed: 1) <i>Tetraselmis chuii</i> 2) <i>Phaeodactylum tricornutum</i> 30 days	Not evaluated	↑ Oedema and inflammation signs ↓ Villus height ↓ Goblet cells	↓ Richness and diversity	Not evaluated	Cerezuela et al. (2012a)

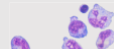
Fish Species and Weight 	Algal inclusion in diet and Trial Duration 	Growth Performance 	Intestinal Morphology and Functionality 	Intestinal Microbiota Structure and Composition 	Immune Response and Disease Resistance 	Reference 
Gilthead seabream 100 g	Microalgae inclusion at 50 and 100 g kg ⁻¹ feed: 1) <i>Nannochloropsis gaditana</i> 2) <i>Tetraselmis chuii</i> 3) <i>Phaeodactylum tricornutum</i> 2 or 4 weeks feeding trial	↔ FBW	Not evaluated	Not evaluated	Algae supplementation modulated the immune response in the head-kidney: ↑ Haemolytic complement activity (<i>N. gaditana</i> and <i>T. chuii</i> 100 at 2 weeks; <i>P. tricornutum</i> at 2 and 4 weeks) ↑ Phagocytic ability (<i>P. tricornutum</i> at 2 weeks) ↑ Phagocytic capacity (<i>N. gaditana</i> 100 and <i>P. tricornutum</i> at 2 weeks; <i>N. gaditana</i> 50, <i>T. chuii</i> and <i>P. tricornutum</i> 100 at 4 weeks) ↑ Respiratory burst (<i>P. tricornutum</i> at 2 weeks) ↑ Expression of β -defensin gene (<i>N. gaditana</i> 100 at 4 weeks) ↑ Expression of <i>mhcIIa</i> and <i>csf-1r</i> genes in gut (<i>T. chuii</i> 50)	Cerezuela et al. (2012b)
Gilthead seabream 8 g	Microalgae <i>Scenedesmus almeriensis</i> inclusion at 12, 20, 25 and 39% in feed (partially replacing FM) 45 days	↔ FBW	↑ Villus length (20, 25 and 39% inclusion) ↑ Enterocytes height (25% inclusion) ↑ Microvillus length, enterocyte apical area and absorption surface (20% inclusion) ↑ Microvillus diameter ↓ Microvillus length (12 and 25% inclusion) ↑ Digestive capacity: ↑ Trypsin activity (12% inclusion) ↑ Leucine aminopeptidase and alkaline phosphatase (20% inclusion)	Not evaluated	Not evaluated	Vizcaíno et al. (2014)

Fish Species and Weight 	Algal inclusion in diet and Trial Duration 	Growth Performance 	Intestinal Morphology and Functionality 	Intestinal Microbiota Structure and Composition 	Immune Response and Disease Resistance 	Reference 
Gilthead seabream 14 g	Macroalgae inclusion at 5, 15 and 25% in feed (partially replacing FM): 1) <i>Gracilaria cornea</i> 2) <i>Ulva rigida</i> 70 days	↑ FBW only in <i>U. rigida</i> at 25% inclusion ↔ FBW all other treatments	Not evaluated	↑ Richness at 15% inclusion both algae ↓ Richness at 25% inclusion of <i>U. rigida</i> ↑ <i>Pseudomonas</i> (<i>G. cornea</i> 15) ↑ <i>Lactobacillus delbrueckii</i> subspecies (<i>G. cornea</i> 25) ↑ <i>Lactobacillus</i> and <i>Sphingomonas</i> (<i>U. rigida</i> 5 and 15) ↓ <i>Vibrio</i> and loss of <i>Photobacterium</i> as dominant genus (<i>G. cornea</i>)	Not evaluated	Rico et al. (2016)
Gilthead seabream 57 g	Microalgae <i>Nannochloropsis gaditana</i> inclusion at 5, 7.5 and 15 g kg ⁻¹ (partially replacing cellulose) 37 days	↔ FBW	↔ Intestinal mucosa and digestive capacity	↔ Richness (higher values at 7.5 inclusion but only significant compared to lowest inclusion level)	↔ Expression of markers of inflammation (<i>il-1β</i> and <i>tnf-α</i>) ↔ Expression of genes encoding enzymes involved in redox homeostasis (CuZnSOD, MnSOD and CAT)	Jorge et al. (2019)
Gilthead seabream 14 g	Macroalgae <i>Ulva rigida</i> inclusion at 25% (partially replacing FM) 30 days followed by infection trial with <i>Photobacterium damsela</i> subsp. <i>piscicida</i> (Phdp)	↔ FBW	Not evaluated	↑ Richness ↑ <i>Lactobacillus brueckii</i> ↓ <i>Photobacterium</i>	↔ Respiratory burst activity ↑ ACH50 activity ↑ Mortality (Phdp infection)	Abdala-Díaz et al. (2021)

Fish Species and Weight 	Algal inclusion in diet and Trial Duration 	Growth Performance 	Intestinal Morphology and Functionality 	Intestinal Microbiota Structure and Composition 	Immune Response and Disease Resistance 	Reference 
Largemouth bass (<i>Micropterus salmoides</i>) 212 g	Microalgae <i>Chlorella vulgaris</i> inclusion at 74% in feed (replacing all FM) 8 weeks	Not evaluated	↓ Villus height ↑ Intestinal structure (mucosal layer) ↓ MUC2, IL-1 β , TNF- α and MDA activities ↓ Expression of <i>il-1β</i> , <i>tnf-α</i> , <i>il-10</i> and <i>tgf-β</i> ↑ Claudin-1, GSH-Px and Nf-2 activities and expression of <i>claudin-1</i>	↑ <i>Lactobacillus</i> , <i>Bacillus</i> , and <i>Enterococcus</i> ↑ Intestinal short fatty acids, namely acetic acid ↓ Pathogenic bacteria	↔ Plasma IgG, IgM, and IL-1 β	Li et al. (2023)
Nile tilapia (<i>Oreochromis niloticus</i>) 24 g	Microalgae <i>Chlorella vulgaris</i> inclusion at 10% in feed Exposure to herbicide penoxsulam followed by 15- or 30-days feeding trial	↑ FBW	Not evaluated	Not evaluated	Microalgae supplementation provided protection against penoxsulam-induced immunotoxicity: ↑ Phagocytic index ↑ Plasma lysozyme, anti-trypsin activity bactericidal activity ↑ Expression of <i>il-1β</i> and <i>tnf-α</i> in spleen ↑ Oxidant/antioxidant and liver status ↑ Resistance to <i>Aeromonas sobria</i> infection	Galal et al. (2018)
Rainbow trout (<i>Oncorhynchus mykiss</i>) 31 g	Microalgae <i>Schizochytrium limacinum</i> inclusion at 5% (partially replacing FO) 15 weeks	↔ FBW	Not evaluated	↔ Community structure ↑ Diversity ↑ <i>Streptococcus</i> , <i>Leuconostoc</i> , <i>Lactobacillus</i> , <i>Lactococcus</i> and <i>Weissella</i>	Not evaluated	Lyons et al. (2017)
Rainbow trout 67 g	Macroalgae <i>Gracilaria vermiculophylla</i> inclusion at 5 and 10% in feed (partially replacing FM and other protein sources) 91 days	↓ FBW (10% inclusion)	↓ Intestine diameter (10% inclusion) ↔ Villus height	Not evaluated	↑ Lysozyme and ACH50 and peroxidase (5% inclusion)	Araújo et al. (2016)

Fish Species and Weight 	Algal inclusion in diet and Trial Duration 	Growth Performance 	Intestinal Morphology and Functionality 	Intestinal Microbiota Structure and Composition 	Immune Response and Disease Resistance 	Reference 
Rainbow trout 68 g	Microalgae <i>Chlorella sorokiniana</i> inclusion at 9, 18, 27 and 36% in feed (partially or completely replacing FM) 8 weeks	↑ FBW (36% inclusion)	Microalgae inclusion at 36%: ↑ Villus height and muscularis thickness ↑ Expression of tight junction protein genes <i>cld1</i> , <i>ocln</i> , <i>tric</i> , and <i>zo-1</i> in intestine	↔ Richness and diversity ↑ <i>Lactobacillus</i> ↓ <i>Acinetobacter</i>	↔ Blood metabolites Microalgae inclusion at 36%: ↑ Expression of immune-related genes <i>il-1β</i> , <i>il-8</i> , <i>tgf-β</i> , <i>tnf-α</i> and <i>igt</i> in intestine ↑ Expression of mTOR pathway-related genes <i>akt1</i> , <i>mtor</i> , and <i>eif4ebp</i> and <i>rps6k1</i> in liver	Liu et al. (2022a)
Rockfish (<i>Sebastes schlegelii</i>) 8 g	Macroalgae <i>Codium fragile</i> -derived sulfated polysaccharides inclusion at 0.1, 0.5 and 1% in feed (partially replacing cellulose) 2 or 4 weeks followed by infection trial with <i>Edwardsiella tarda</i>	Not evaluated	Not evaluated	Not evaluated	↓ Expression of cytokines in spleen ↓ Expression of <i>il-1β</i> gene in intestine (2 weeks for 0.5% group) ↑ Expression of <i>il-10</i> gene (2 weeks for 0.5 and 1% groups; 4 weeks for 0.5% group) Post- <i>E. tarda</i> infection - 0.1% group: ↓ Expression of <i>il-1β</i> and <i>il-8</i> genes in head-kidney (day 3) ↓ Mortality Post- <i>E. tarda</i> infection - 0.5% group: ↑ Expression of <i>il-1β</i> and <i>il-6</i> genes in head-kidney (day 1) ↓ Expression of <i>il-1β</i> in liver (day 3)	Yang et al. (2021)

Fish Species and Weight 	Algal inclusion in diet and Trial Duration 	Growth Performance 	Intestinal Morphology and Functionality 	Intestinal Microbiota Structure and Composition 	Immune Response and Disease Resistance 	Reference 
Senegalese sole (<i>Solea senegalensis</i>) 23 g	Macroalgae inclusion at 10% in feed (partially replacing soybean and wheat meal): 1) <i>Ulva rigida</i> 2) <i>Undaria pinnatifida</i> 5 months (juvenile phase) 9 months (on-growing stage)	<i>U. rigida</i> : ↔ FBW <i>U. pinnatifida</i> : ↔ FBW (juvenile phase) ↓ FBW (on-growing phase)	<i>U. rigida</i> : ↔ Gut morphology <i>U. pinnatifida</i> : ↓ Villus width	Not evaluated	Not evaluated	Moutinho et al. (2018)
Senegalese sole 11 g	Macroalgae <i>Ulva ohnoi</i> inclusion at 5% (partially replacing FM) 45 days	↓ FBW	Not evaluated	↔ Richness Anterior intestine: ↑ Diversity ↑ <i>Vibrio</i> ↓ <i>Escherichia</i> and <i>Tenacibaculum</i>	Not evaluated	Tapia-Paniagua et al. (2019)
Senegalese sole 11 g	Macroalgae <i>Ulva ohnoi</i> inclusion at 5% (partially replacing FM) 45 or 90 days	↓ FBW	↑ Villus height, enterocytes height and total enterocyte absorption surface (45 days) ↑ Alkaline phosphatase ↓ Total alkaline protease, trypsin and chymotrypsin	Not evaluated	↔ Expression of genes related to stress in liver	Vizcaíno et al. (2019)
Senegalese sole 11 g	Macroalgae <i>Ulva ohnoi</i> inclusion at 5% (partially replacing FM) 90 days followed by infection trial with <i>Photobacterium damsela</i> subsp. <i>Piscicida</i> (Phdp)	↓ FBW	Not evaluated	Not evaluated	Algae supplementation modulated the liver immune response after infection: ↑ Expression of immune-related genes <i>c1q4</i> , <i>c3</i> , <i>c9</i> , <i>lysg</i> , <i>tnf-α</i> ↑ Expression of antioxidant response genes <i>gpx</i> and <i>sodmn</i> ↑ Expression of iron metabolism genes <i>ferrm</i> and <i>hamp-1</i> ↓ Expression of inflammatory cytokines <i>il-1β</i> , <i>il6</i> and <i>il-8β</i> ↓ Pathogen loads	Fumanal et al. (2020)

Fish Species and Weight 	Algal inclusion in diet and Trial Duration 	Growth Performance 	Intestinal Morphology and Functionality 	Intestinal Microbiota Structure and Composition 	Immune Response and Disease Resistance 	Reference 
Senegalese sole 11 g	Macroalgae <i>Ulva ohnoi</i> inclusion at 5% (partially replacing FM) 90 days	↓ FBW	Not evaluated	↔ Richness and diversity ↑ <i>Vibrio</i> ↓ <i>Stenotrophomonas</i> ↑ Predicted genes involved in xenobiotic biodegradation ↓ Predicted genes associated to penicillin and cephalosporin biosynthesis and beta-lactam resistance	Not evaluated	Cerezo et al. (2022)
Zebrafish (<i>Danio rerio</i>) Larvae	Microalgae inclusion at 10 g kg ⁻¹ feed (included in FM- and soybean meal-based diets): 1) <i>Tetraselmis</i> sp. 2) <i>Phaeodactylum tricornutum</i> 3) <i>Chlorella</i> sp. 4) <i>Nannochloropsis oculata</i> 5) <i>Nannochloropsis gaditana</i> 5 to 8 dpf followed by infection trial with <i>Edwardsiella tarda</i>	Not evaluated	Soybean meal-based diet supplemented with algae: ↓ Neutrophils compared to only soybean group	Not evaluated	Both FM- and soybean meal-based diets supplemented with algae: ↓ Mortality in <i>Tetraselmis</i> sp. and <i>Chlorella</i> sp. groups (<i>E. tarda</i> infection)	Bravo-Tello et al. (2017)

1.4 Emerging approaches to evaluate the impact of functional diets on fish health

The extensive use of animals for experimentation has raised ethical concerns, leading scientists to re-evaluate traditional testing methods. This shift began in the 1950s with the introduction of the 3R principle, which emphasizes the reduction of animal usage, refinement of procedures to minimize pain and stress, and the replacement of animals with non-sentient materials, as outlined by Russell and Burch (1960).

The development of *in vitro* methods presents an alternate approach in aquaculture research to investigate fish health, diagnose diseases and improve nutrition (Goswami et al., 2022). In today's context, this effort is particularly significant given that millions of animals are used for experimentation in the European Union, resulting in costs amounting to billions of euros (European Commission, 2023). Furthermore, there is a growing societal pressure to minimize animal usage in research due to welfare concerns, which has led to the regulation of European animal experimentation by Directive 2010/63 EU since 2013 (Olsson et al., 2017). The imperative for discovering novel ingredients and additives to bolster fish robustness in light of intensified farming practices is of utmost importance in aquaculture research (Boyd et al., 2020). However, testing new compounds for aquafeeds still heavily relies on live animal trials that are time-consuming, expensive, and increasingly constrained by animal experimentation regulations (Figure 3). Establishing state-of-the-art fish *in vitro* models can significantly reduce the reliance on animal-based *in vivo* experiments and associated costs, allowing the aquaculture sector to explore alternative feed formulations more quickly, cost-effectively, and ethically (Goswami et al., 2022).

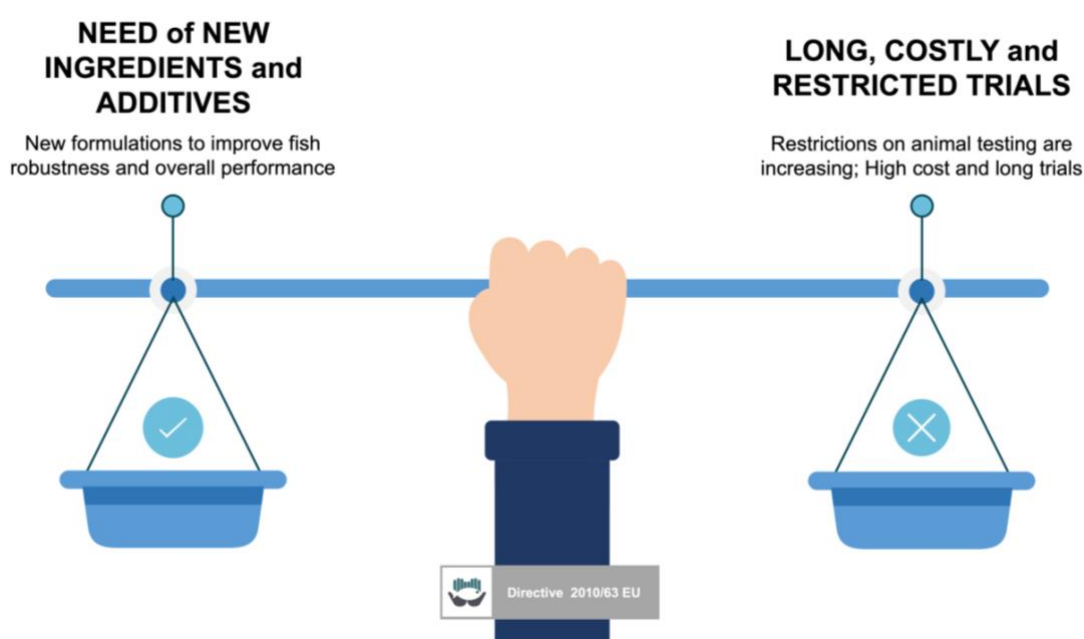


Figure 3. Overview of needs and limitations in aquaculture research.

Cell cultures are also promising tools for studying defence mechanisms and immunological systems of fish when exposed to various external stressors (Goswami et al., 2022). They provide controlled and defined physicochemical environments, reducing assay time and allowing for direct cell exposure to compounds. However, they have some disadvantages, including inherent tissue sample heterogeneity and the simplified cellular environment, which may not fully replicate *in vivo* interactions (Astashkina et al., 2012; Freshney, 2010; Segeritz & Vallier, 2017). *In vitro* tools often involve primary cells and cell lines. Primary cells closely resemble *in vivo* cellular dynamics but have limited viability over time. These cells are prepared directly from dispersed cells derived from specific tissues or organs, allowing for monolayer formation and subsequent subculturing to potentially create spontaneously immortalized cell lines (Astashkina et al., 2012; Eglen & Reisine, 2011). Most studies on fish immune cells have primarily focused on monocyte-derived macrophage-like cells from haematopoietic organs such as the head-kidney and spleen (Iliev et al., 2019; Iliev et al., 2013; Joerink et al., 2006). However, when it comes to European seabass, limited research has been conducted on the development of *in vitro* tools for investigating its immune system. Leucocytes from the head-kidney have been isolated in some studies to assess the impact of amino acid supplementation (Azeredo et al., 2017b; Machado et al., 2021) or contaminants (Espinosa et al., 2018; Morcillo et al., 2015; Morcillo et al., 2016) on the fish's immune system. Despite the acknowledged importance of GALTs in fish immunity, there is still a significant knowledge gap regarding the immune cell populations within the fish intestine. The advancement of research on intestinal immune cells is hindered by the absence of suitable cell markers and the complexity of isolation protocols. While isolation protocols for intestinal cells have been described for some fish species like rainbow trout (Bernard et al., 2006), gilthead seabream (Salinas et al., 2007) and Atlantic salmon (Park et al., 2020b), the European seabass remains relatively unexplored in this regard.

To bridge this gap, the potential of high-throughput techniques such as imaging flow cytometry (IFC) and RNA sequencing are being explored. In a recent study on Atlantic salmon, transcriptomic techniques were employed on adherent cells from the distal intestine, revealing a high expression of macrophage-related genes in this cell population (Park et al., 2021; Park et al., 2020b). These findings emphasize the potential of next-generation methods to provide vital insights into the characteristics and functions of cell populations within the fish intestine. In the case of European seabass, various cell populations, including T and B cells, macrophages, and granulocytes, are found in the GALT (Picchietti et al., 2021b). Previous research has indicated the expression of marker genes for T cells in the European seabass GALT, such as *rag-1*, *cd3ε*, *tcrβ*, *cd8α*, and *cd4* (Boschi et al., 2011; Picchietti et al., 2021a). Nevertheless, there is a scarcity of in-depth

knowledge regarding the characteristics and functions of the different immune cell populations in the European seabass intestine.

Overall, the limited understanding of the intestinal immune system in fish compared to mammals continues to pose a barrier to the development of efficient tools for enhancing fish immunity. Novel *in vitro* tools can offer valuable insights into how immune cell populations function and contribute to shaping the farmed fish's immune system, particularly in the context of diseases (Goswami et al., 2022). By advancing our fundamental knowledge of fish immune responses, with a specific focus on gut immune cells and their functions, innovative solutions may be uncovered to boost fish immunity and effectively mitigate the impact of disease outbreaks. This progress can, in turn, contribute to the enhancement of commercial fish production efficiency, promote sustainability within the aquaculture sector, and align with ethical considerations in animal welfare.

1.5 Objectives of the thesis

The increase in the world population combined with the shift in consumer trends towards healthier and natural eating habits places pressure on the aquaculture sector to meet the high demand for fish. The intensification of fish farming is, however, often associated with a rise in disease outbreaks, with bacterial diseases caused by Gram-negative bacteria (e.g., *Tenacibaculum maritimum*) being responsible for high mortality rates and devastating economic losses. Antimicrobial resistance has been recognized as a critical problem in the past years. Hence, there is a need for judicious use of antibiotics to treat diseases in animals (Kasimanickam et al., 2021). In this context, innovative approaches to improve fish robustness and welfare are of utmost importance, to ensure aquaculture profitability. Given the intimate correlation between nutrition and health, it is imperative that new functional supplements, especially those derived from algae, receive special attention in the realm of aquaculture research.

Algae are rich sources of bioactive compounds and their inclusion in aquafeeds has been explored in the past years. Several studies have demonstrated the potential of algae to enhance immune response and improve disease resistance in fish. The intestinal microbiota also plays a key role on host health, affecting fish immunity and overall performance. As diet significantly influences not only the composition and diversity of microbial communities in the intestine but also its overall morphology and physiology, incorporating algae into aquafeeds holds the potential to modulate the intestinal health of farmed fish. However, there are not many studies on the manipulation of intestinal health through dietary inclusion of ingredients such as algae, with inherent added-value on immunity and overall fish health.

Therefore, the main goal of this thesis is to better understand the potential of algae to improve farmed fish robustness through dietary modulating of intestinal health. To achieve this goal, first, I performed a chemical and functional characterisation of commercially available micro- and macroalgae species, to identify their potential to be included in aquafeeds (Chapter 2). Then, I addressed the impact of selected algae on the intestinal microbiota and structure of European seabass, to uncover the ability of these added-value products to modulate the microbial communities present in fish intestine (Chapters 3 and 4). To better understand the potential of such algae to improve fish robustness, European seabass was subjected to a bacterial challenge following a feeding trial with algae feeds to evaluate both systemic and mucosal immune responses (Chapter 5). Finally, I explored new approaches to improve and /or develop new methods for assessing the intestinal morphology and immune responses of European seabass, with the aim of providing novel tools for future studies targeting the modulation of fish intestinal health through dietary interventions (Chapters 6 and 7).

Chapter 2.

Nutritional value, antimicrobial and antioxidant activities of micro- and macroalgae, single or blended, unravel their potential use for aquafeeds

By

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Abstract

Algae are natural products with great potential as aquafeed ingredients, being rich in nutrients and bioactive compounds. They can improve fish health while being sustainable at social, economic, and environmental levels, contributing to the one health concept. In this study, two micro- (*Nannochloropsis oceanica* and *Chlorella vulgaris*) and two macroalgae (*Gracilaria gracilis* and *Ulva rigida*), produced under commercial conditions, were selected to unravel their nutrition value (protein and lipid content; fatty acid and amino acid profiles), as well as antimicrobial activity against farmed fish and shrimp pathogenic bacteria and bioactive potential by assessing ABTS^{••} and DPPH[•] scavenging capacities. A commercial blend of these algae (ALGAESSENCE[™] – Feed) was included to determine possible synergistic effects. *N. oceanica* was rich in eicosapentaenoic acid and arachidonic acid (ARA) and *G. gracilis* had high content of ARA. *C. vulgaris* had the highest levels of essential amino acids (EAAs), namely lysine. The blend is a well-balanced and rich source of proteins, lipids, essential fatty acids, EAAs and carbohydrates. The single algae and the blend displayed bactericidal and bacteriostatic activities against most of the tested pathogenic bacteria, with the most promising results being observed against *Tenacibaculum maritimum* (40–45% activity). In some cases, the micro- and macroalgae had no simultaneous bactericidal and bacteriostatic activities, but the blend was able to both kill and inhibit the growth of those bacteria. The algae had also some antioxidant activity, with *G. gracilis* and the blend presenting the highest values. The present results showcased the blend as a promising ingredient to be included in aquafeeds.

Keywords: Bioactive potential, Functional feeds, One health, Microalgae, Seaweeds, Sustainable marine products

2.1 Introduction

Optimization of aquafeeds is a subject of particular interest for the aquaculture sector, and efforts have been made to produce novel functional diets capable of promoting both fish growth and health, while being sustainable at social, economic and environmental levels (Encarnação, 2016), contributing to the one health concept. In this context, it is imperative to have an in-depth comprehension of the nutritional value and *in vitro* bioactive potential of sustainable marine products, namely micro- and macroalgae, in order to select the best candidates to be included in aquafeeds.

The increasing interest in microalgae is not only related to their high protein contents (40-70%) and balanced amino acids profiles but also their valuable sources of lipids (i.e. long-chain polyunsaturated fatty acids, LC-PUFA) and bioactivity (Shah et al., 2018). In recent years, we have observed an increase in demand for both functional feed and food, namely microalgae and their derivate. Indeed, about 30% of microalgal production is currently directed towards animal feed applications (Hamidi et al., 2019). The microalgae *Nannochloropsis oceanica* and *Chlorella vulgaris* are of particular relevance to be used in aquaculture (Batista et al., 2020a; Raji et al., 2018; Sørensen et al., 2017). *Nannochloropsis* sp. are small-sized green algae specifically known for both their high-quality protein and content of the fatty acid eicosapentaenoic acid (EPA) (Hemaiswarya et al., 2011). LC-PUFAs like EPA are appreciated for their nutritional value and associated health benefits; they are relevant for marine fish that are unable to synthesize such essential nutrients (Tocher, 2010). EPA is involved in several physiological and immune processes, and its content in aquafeeds can likely be translated into both fish health and omega-3 fatty acids-rich fillets for human consumption (Tocher, 2015). *Nannochloropsis* sp. are also valuable sources of carotenoids (i.e., zeaxanthin, canthaxanthin and astaxanthin), carbohydrates and other compounds with possible immunostimulatory effects capable of improving fish health when supplemented in feeds (Cerezuela et al., 2012b; Shah et al., 2018). *Chlorella* is among the microalgae genera with the highest production volumes, with a worldwide production of 5000 tons per year. *Chlorella* sp. have high protein content (up to 60%) and produce a wide range of compounds, namely carotenoids, β -1,3-glucan, vitamins, and polyphenols with, among others, antioxidant and antimicrobial activities (García et al., 2017; Hamidi et al., 2019).

Macroalgae, commonly denominated as seaweeds, have already several commercial applications; human and animal nutrition, agricultural purposes (fertiliser industry), and biofuel production (Ghadiryannfar et al., 2016; Tiwari & Troy, 2015). Among seaweeds, red and green algae, such as *Gracilaria gracilis* and *Ulva rigida*, have instigated the interest of the fish feed sector due to their capacity to achieve high yields of biomass and the

production of biologically active phytochemicals (Bruhn et al., 2011; Capo et al., 1999; Francavilla et al., 2013). As recognised edible seaweeds, whole *G. gracilis* and *U. rigida* have been commonly used as food. On the other hand, their polysaccharide fractions are also potential valuable products, with agar from *G. gracilis* being already used for food, pharmaceutical and biotechnological applications (Torres et al., 2019). In contrast, the polysaccharide ulvan, a cell wall component of *U. rigida*, remains underexplored despite the broad range of biological activities displayed by this compound (e.g. immunostimulant, antioxidant, anti-viral, anticancer and anticoagulant) (Kidgell et al., 2019). *Gracilaria* sp., namely *G. gracilis*, are rich sources of arachidonic acid (ARA), with values ranging from 16 to 48% total fatty acids, depending on the season (Francavilla et al., 2013). ARA is an essential PUFA for marine fish, due to its role as an eicosanoid precursor, being involved in several physiological processes, including immunity and stress response (Tocher, 2003; Torrecillas et al., 2017b). Despite the highly variable nutritional value of red and green seaweeds, they can be good sources of proteins, and when produced through integrated multitrophic aquaculture (IMTA) systems their protein contents can reach 30-50%, DM basis (Abreu et al., 2011; Shpigel et al., 2017).

Algae produce a wide variety of metabolites with potential antibacterial and antioxidant activities; their contents of pigments (e.g. carotenoids), polyphenols, polysaccharides, sterols, and vitamins are of particular interest for the feed industry (Abu-Ghannam & Rajauria, 2013). Algal compounds with antioxidant properties are able to counteract the oxidative damage caused by free radicals through direct neutralization of these radicals, by interfering with the expression or activity of free radical-generating enzymes or by promoting an increased expression of the endogenous antioxidant enzymes (Lü et al., 2010). On the other hand, these compounds exert their antimicrobial action through two possible mechanism: bactericidal activity, by direct destruction of the microorganism (e.g. alter bacteria cell wall permeability, thereby causing the loss of cellular integrity); or bacteriostatic activity, by inhibiting the growth of the microorganisms (Abu-Ghannam & Rajauria, 2013). The *in vitro* antimicrobial and antioxidant activities of extracts from representative species of Chlorophyta, Rhodophyta and Phaeophyta have been previously demonstrated (Bansemir et al., 2006; Chew et al., 2008; Narasimhan et al., 2013). *In vivo*, nutritional studies have also been performed on relevant farmed fish species, such as European seabass (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*) and rainbow trout (*Oncorhynchus mykiss*), to unravel the impact of seaweed supplementation; beneficial effects were reported in terms of immune and antioxidant status, as well as resistance to stress and bacterial diseases (Araújo et al., 2016; Magnoni et al., 2017; Peixoto et al., 2019). Likewise, the inclusion of microalgae, namely *Chlorella* sp., in aquafeeds has been previously associated with additional health benefits for farmed fish species that included

stimulation of immune response and improved antioxidant status (Cerezuela et al., 2012b; El-Habashi et al., 2019; Messina et al., 2019; Teimouri et al., 2019). Although most nutritional studies in fish are focused on the potential of a single algae species to be included in aquafeeds (Bravo-Tello et al., 2017; Cerezuela et al., 2012b; Fumanal et al., 2020; Teimouri et al., 2019; Vizcaíno et al., 2016; Wassef et al., 2013), the simultaneous inclusion of two or more micro- and macroalgae can help achieving balanced and highly nutritional blends, by combining protein sources poor in lipids (macroalgae), with microalgae that have high contents of omega-3 fatty acids. Additionally, a blend may allow synergetic antibacterial and antioxidant activities derived from the distinct species (Amaro et al., 2019). In the present study, two microalgae (*N. oceanica* and *C. vulgaris*) and two macroalgae (*G. gracilis* and *U. rigida*) already produced under commercial conditions were selected to evaluate their nutritional value and unravel their antibacterial and antioxidant capacities. A commercial blend of the four algae (ALGAESSENCE™ – Feed) was also included to evaluate possible synergistic effects of the selected algae. The results obtained herein will help elucidate the *in vitro* potential of these algae to be included in functional aquafeeds and improve the robustness of farmed aquatic animals.

2.2 Materials and methods

Two microalgae algae *N. oceanica* (NAN) and *C. vulgaris* (CHLO), two macroalgae *G. gracilis* (GRA) and *U. rigida* - ULVA) and a commercial blend of these algae (ALGAESSENCE™ – Feed; BLEND) were selected for chemical characterisation and evaluation of their *in vitro* activity. The individual micro- and macroalgae are produced at industrial scale by two companies, Allmicroalgae (Pataias, Portugal) and ALGAplus (Ílhavo, Portugal), respectively. The microalgae were firstly produced in a glass autotrophic reactor and then moved to an industrial tubular reactor placed outdoors. After pasteurization and centrifugation, the algae biomass was spray-dried. The macroalgae were produced in an integrated multi-trophic aquaculture (IMTA) system, at Ria de Aveiro (Portugal), and were dried by convection. The blend (ALGAESSENCE™ – Feed) is a well-balanced commercial mixture of the four algae.

2.2.1 Chemical characterization of the selected algae

The selected algae were chemically analysed, in duplicate, according to AOAC (2006) methods: dry matter (DM) at 105 °C for 24 h; ash, by combustion in a muffle furnace at 500 °C for 5 h; crude protein by a Leco nitrogen analyzer (Model FP-528, Leco Corporation, Michigan, USA) and using $N \times 6.25$; and gross energy using an adiabatic bomb calorimeter

(Werke C2000; IKA, Staufen, Germany). Carbohydrates were calculated by estimation: 100 – (crude protein + crude fat + ash).

Total lipids from each alga were extracted and quantified according to Folch et al. (1957). The fatty acid methyl esters (FAMES) of the algae were produced by direct transesterification by acidic methylation, as described by Lepage and Roy (1986). Firstly, 1 mL of tricosanoic acid (23:0; Sigma-Aldrich, Darmstadt, Germany) at 1 mg mL⁻¹ (diluted in *n*-hexane) was added as internal standard to each sample. After the addition of 3 mL of freshly prepared 5% (v/v) acetyl chloride in methanol, samples were mixed and incubated at 100 °C for 60 min and cooled at room temperature. Potassium carbonate 6% (v/v) (5 mL) was added to each sample and FAMES were recovered in 2 mL of *n*-hexane with BHT. After shaking and a 5 min centrifugation (4,000 × g) at room temperature (RT), the organic phase was transferred to new tubes with 1 g of anhydrous sodium sulphate and 200 mg of activated charcoal (to remove the sample's colour). The addition of the *n*-hexane with BHT and the following steps were repeated two more times. Afterwards, the tubes with the organic phase were shaken vigorously and filtrated to new tubes using Whatman paper filters previously moistened with *n*-hexane and evaporated in a nitrogen stream at 37 °C. The samples were diluted in *n*-hexane and placed in glass vials for posterior ionization detection (GC-FID) analysis. The FAMES analysis was performed using a Shimadzu GC-2010 Plus gas chromatograph (Shimadzu, Tokyo, Japan), coupled with an autoinjector AOC-20i (Shimadzu) and a flame-ionization detector (GC-FID; Shimadzu), with helium as carrier gas. The separation was carried out on an CP-Sil 88 capillary column (50.0 m × 0.25 mm I.D., film thickness 0.20 µm; Middelburg, Netherlands). Column temperature conditions were as follows: 120 °C for 5 min; 2 °C min⁻¹ increase until 160 °C, for 15 min; and 2 °C min⁻¹ increase until 220 °C, for 10 min. Operation conditions included a split mode, with split ratio of 1:50 and an injection volume of 1.0 µL (Nunes et al., 2018), and the temperatures of the injector and detector were, respectively, 250 and 270 °C. FAMES were identified by comparison with known standard mixtures (FAME 37, Supelco, Bellefonte, USA) and each FAME were expressed on the % of total FAMES basis, represented in the chromatogram (Reis et al., 2014). The quantity of fatty acids (mg g⁻¹) was calculated according to Joseph and Ackman (1992).

For amino acid (AA) profile, an acid hydrolysis (6 M HCl) of the algae was performed in an oven for 48 h at 110 °C. After hydrolyses, samples were precolumn derivatized with Waters AccQ Fluor Reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) using the AccQ Tag method (Waters, Milford, USA). Ultra-high-performance liquid chromatography (UPLC) in a Waters Reversed-Phase Amino Acid Analysis System was used for the analysis, with norvaline as an internal standard. The peaks were analysed with EMPOWER software (Waters). Tryptophan was not determined because it is partially destroyed during the acid

hydrolysis. The sum of AAs was used to determine the protein content of the algae (true protein).

2.2.2 Algae *in vitro* bactericidal and bacteriostatic activities

The *in vitro* bactericidal and bacteriostatic activities of the individual algae and the blend were tested against some of the main pathogenic bacteria for farmed aquatic animals (i.e., *Vibrio anguillarum*, *V. harveyi*, *V. parahaemolyticus*, *Aeromonas hydrophila*, *Yersinia ruckeri*, *Edwardsiella tarda*, *Photobacterium damsela* subsp. *piscicida* – Phdp and *Tenacibaculum maritimum*). The antibacterial activities of the algae were evaluated according to Graham et al. (1988) and Sunyer and Tort (1995), with modifications. Briefly, the dried algae (three biological replicates per algae) were dissolved in distilled water to a final concentration of 1 mg mL⁻¹ and homogenised in orbital agitator for 30 min at RT. The samples were then centrifuged for 10 min at 4 °C and 5,000 x g, followed by a filtration of the extracts through a 0.22 µm membrane. Bacteria used in this assay were grown in Tryptone Soy Agar (TSA; Condalab, Madrid, Spain) supplemented with NaCl to a final concentration of 2% (w v⁻¹) or Marine Agar (Condalab) and resuspended in TSB at 2% NaCl or Marine Broth (MB; Condalab). The bacterial suspensions were adjusted to an Absorbance (Abs) at 600 nm of around 0.3, corresponding to 1-3 × 10⁷ colony forming units (cfu) mL⁻¹.

Regarding the bactericidal activity, 20 µL of each algae extract, in triplicate, were incubated with 20 µL of each bacterial suspension in a U-shaped 96-well microplate, for 2.5 h at 25 °C with shaking. The samples were, afterwards, incubated with 25 µL of Thiazolyl Blue Tetrazolium Bromide (Sigma-Aldrich) at 1 mg mL⁻¹, for 10 min at 25 °C with shaking, followed by a 10 min centrifugation at 2,000 x g. The supernatant was discarded, and the pellet obtained was dissolved in 200 µL Dimethyl Sulfoxide (DMSO, Sigma-Aldrich). After complete dissolution, 100 µL of each sample were placed into a flat-bottomed 96-well microplate and the Abs at 560 nm was measured using a Synergy HT microplate reader (Biotek, Vermont, USA). The bactericidal activity was calculated according to the percentage of non-viable bacteria, using the following formulas: % viable bacteria = [(Abs sample) / (Abs positive control)] x 100; % non-viable bacteria = 100 - % viable bacteria.

The bacteriostatic activity was determined by plating into a flat-bottomed 96-well microplate 20 µL of algae extract in triplicate, with 20 µL of each bacterial suspension and 160 µL of TSB at NaCl 2%, followed by an 24 h incubation at 25 °C with shaking. The absorbance (Abs) at 630 nm was measured using a Synergy HT microplate reader. The bacteriostatic activity was calculated as follows: % viable bacteria = {(Abs sample at 24h – initial Abs sample) – Abs blank} / [(Abs positive control at 24h – initial Abs positive control) – Abs

blank)}} x 100; % non-viable bacteria = 100 - % viable bacteria. Due to experimental constraints, namely the formation of bacterial aggregates over the 24 h period, it was not possible to determine the bacteriostatic activity for the bacterium *T. maritimum*.

For both assays, NaCl 0.9% was used instead of the algae extracts as positive control, while for the negative control TSB at NaCl 2% was added instead of the bacterial suspension.

2.2.3 Algae *in vitro* antioxidant capacity: ABTS^{•+} and DPPH[•] scavenging capacities

The *in vitro* antioxidant assays were performed employing the same extracts used to test the bactericidal and bacteriostatic activities of the algae. The radical-scavenging potential of the algae extracts was determined through the 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) radical cation (ABTS^{•+}) assay, according to Amaro et al. (2019). The ABTS^{•+} solution was prepared from ABTS (Sigma-Aldrich) and K₂S₈O₂ (Sigma-Aldrich), as described by Guedes et al. (2013). Triplicate samples of each extract (63 µL) were resuspended in ethanol in a flat-bottomed 96-well plate, followed by the addition of 180 µL of ABTS^{•+}. The Abs at 734 nm was measured after a 6 min incubation at RT in the dark.

The antioxidant capacity of the extracts was obtained through the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical (DPPH[•]) assay, as described by Amaro et al. (2015). Briefly, 63 µL of each extract resuspended in methanol were incubated with 180 µL of DPPH[•] in a flat-bottomed 96-well plate, for 30 min at RT in dark. The scavenging reaction was determined by measuring the Abs at 515 nm at the end of the incubation period.

For both ABTS^{•+} and DPPH[•] assays, the % of inhibition was calculated as follows: % inhibition = [(Abs ABTS^{•+} or DPPH[•] - Abs sample) / Abs ABTS^{•+} or DPPH[•]] x 100. Trolox (Acros Organics, Thermo Fisher Scientific, Massachusetts, United States) was used as the standard to produce a calibration curve, and the results were expressed as Trolox Equivalent (TE) per unit of biomass (dry weight, DW).

2.2.4 Statistical analysis

Statistical analyses were performed using SPSS 26.0 (SPSS Inc., Chicago, IL, USA). Data were analysed for normal distribution and homogeneity of variances with Kolmogorov-Smirnov and Levene's tests, respectively, and transformed whenever necessary. If normality and/or homogeneity of variances assumptions were not met after transformation, a non-parametric test (Kruskal-Wallis H-test) was used. To compare individual means, one-way ANOVA followed by Tukey's multiple comparison test was performed.

2.3 Results

2.3.1 Nutritional value of the selected algae species

The proximal composition and amino acid profile of the tested algae (*N. oceanica*, *C. vulgaris*, *G. gracilis*, *U. rigida* and the commercial blend of the four algae) are presented in Table 1. In terms of dry matter basis, and using a nitrogen-to-protein conversion factor (Kp) of 6.25, the highest protein content was found in CHLO (57.7%), followed by the BLEND and NAN (36.7 and 34.4%, respectively). The true protein content calculated as the sum of the individual AAs followed this very same trend: 56.7% for CHLO; 34.6% for NAN and 36.9% for the BLEND. True protein values were very close to those estimated by using the Kp of 6.25 (crude protein). NAN had the highest ash content (31.8%), followed by the macroalgae (30.3% for ULVA and 26.4% for GRA) and the BLEND (24.4%). CHLO presented the lowest ash content, with only 9.8%. Concerning carbohydrates, the highest values were found in macroalgae (52.5% for GRA and 45.9% for ULVA), followed by the BLEND at 34.1%. NAN and CHLO had the lowest carbohydrate content, 27.0 and 26.2%, respectively. The gross energy ranged from 11.0 (ULVA) to 21.1 kJ g⁻¹ (CHLO), with the blend presenting an intermediate value of 15.7 kJ g⁻¹. GRA and ULVA had the lowest protein contents (19.6 and 22.1%, respectively) and the lowest essential amino acid levels (9%). The amino acid profile showcased CHLO as the best source of essential amino acids (28.6%, being particularly rich in lysine, 6.7%), followed by the BLEND (18.4%) and NAN (16.6%). The microalgae presented the highest total lipid content: 6.8% for NAN and 6.4% for CHLO. The macroalgae had a low lipid content: 1.5% for GRA and 1.6% for ULVA. The algae blend had an intermediate level (4.8%) of lipids.

Concerning the fatty acid profile (% total fatty acids), the two microalgae had similar levels of saturated fatty acids (SFAs, 33-35%), mainly represented by 16:0 (palmitic acid, 27%). Comparing the two microalgae, NAN had a higher level of 14:0 (myristic acid, 6% vs 1%), while CHLO had higher levels of 18:0 (stearic acid, 3% vs 0.4%). The content of SFAs was very high in both macroalgae (64% in GRA and 75% in ULVA) as a result of the high levels of palmitic acid in ULVA (55%) and both myristic and palmitic acids in GRA (23 and 40%, respectively). The blend of algae had an intermediate level of SFAs content (40%). NAN had the highest levels of monounsaturated fatty acids (MUFAs, 38%), followed by the BLEND (26%), ULVA (13%), GRA (10%) and CHLO (8%). For NAN, ULVA and the BLEND, the 16:1 represented the majority of the MUFAs (32, 11 and 21%, respectively). The microalgae CHLO had the highest contents of polyunsaturated fatty acids (PUFAs), due to the high levels of both 18:2 n-6 c (linoleic acid, 24%) and 18:3 n-3 (α -linolenic acid, ALA, 35%). The lowest levels of PUFAs were found in ULVA (12%), with 3% of linoleic acid and 9% of ALA. NAN and GRA had similar total levels of PUFAs (28 and 26%, respectively),

and were the only species containing LC-PUFA. NAN was rich in 20:5 n-3 (eicosapentaenoic acid, EPA, 16%), while GRA was rich in 20:4 n-6 (arachidonic acid, ARA, 22%). The total PUFAs of the blend was 34%, mainly constituted by ALA (11%), linoleic acid (9%), ARA (7%) and EPA (7%) (Table 2).

Table 1. Single algae and commercial blend proximate composition and amino acid profile (% or kJ g⁻¹ dry matter basis).

	NAN	CHLO	GRA	ULVA	BLEND
Proximal composition (% DM)					
Dry Matter	95.14 ± 2.55	94.75 ± 0.71	92.32 ± 0.11	87.16 ± 0.01	94.11 ± 0.29
Crude Protein (N x 6.25)	34.39 ± 0.09	57.67 ± 0.1	19.58 ± 0.05	22.13 ± 0.08	36.69 ± 0.003
True Protein (Σ AA¹)	34.61 ± 0.13	56.66 ± 0.35	18.70 ± 0.21	21.18 ± 0.09	36.86 ± 0.32
Crude Fat	6.82 ± 0.48	6.36 ± 0.17	1.51 ± 0.10	1.63 ± 0.10	4.83 ± 0.33
Ash	31.83 ± 0.03	9.79 ± 0.002	26.40 ± 0.07	30.34 ± 0.04	24.34 ± 0.06
² Carbohydrates	26.96 ± 0.60	26.19 ± 0.05	52.51 ± 0.22	45.90 ± 0.02	34.14 ± 0.27
Gross Energy, kJ g⁻¹	17.18 ± 0.02	21.06 ± 0.09	13.69 ± 0.06	11.03 ± 0.28	15.69 ± 0.08
Amino acids (% DM)					
³ EAA	16.63 ± 0.08	28.58 ± 0.25	8.54 ± 0.12	9.29 ± 0.07	18.42 ± 0.23
Arginine	2.80 ± 0.01	4.54 ± 0.02	1.46 ± 0.06	1.51 ± 0.03	3.06 ± 0.10
Histidine	0.11 ± 0.003	1.04 ± 0.07	0.17 ± 0.009	0.23 ± 0.001	0.60 ± 0.004
Lysine	2.43 ± 0.03	6.74 ± 0.15	1.34 ± 0.3	1.24 ± 0.05	3.18 ± 0.09
Threonine	1.91 ± 0.01	2.92 ± 0.005	1.04 ± 0.04	1.10 ± 0.01	2.07 ± 0.10
Isoleucine	1.70 ± 0.02	2.19 ± 0.09	0.95 ± 0.05	0.94 ± 0.02	1.67 ± 0.01
Leucine	2.87 ± 0.05	4.25 ± 0.14	1.27 ± 0.05	1.45 ± 0.02	2.83 ± 0.06
Valine	2.06 ± 0.05	3.21 ± 0.06	1.05 ± 0.04	1.22 ± 0.01	2.17 ± 0.14
Methionine	0.66 ± 0.01	1.00 ± 0.06	0.32 ± 0.01	0.27 ± 0.02	0.71 ± 0.02
Phenylalanine	2.08 ± 0.002	2.67 ± 0.005	0.93 ± 0.04	1.23 ± 0.01	2.14 ± 0.02
⁴ CEAA	2.49 ± 0.03	3.01 ± 0.08	0.96 ± 0.04	1.14 ± 0.01	2.21 ± 0.06
Cystine	0.06 ± 0.005	0.20 ± 0.004	0.07 ± 0.003	0.07 ± 0.002	0.13 ± 0.007
Proline	2.43 ± 0.03	2.81 ± 0.08	0.90 ± 0.04	1.08 ± 0.01	2.09 ± 0.06
⁵ NEAA	15.49 ± 0.10	25.08 ± 0.24	9.20 ± 0.17	10.75 ± 0.06	16.22 ± 0.22
Alanine	2.35 ± 0.07	3.94 ± 0.1	1.26 ± 0.05	1.78 ± 0.03	2.67 ± 0.02
Tyrosine	1.59 ± 0.07	2.39 ± 0.08	0.67 ± 0.03	0.86 ± 0.004	1.69 ± 0.10
Aspartate	2.78 ± 0.02	5.40 ± 0.02	2.13 ± 0.09	2.87 ± 0.03	3.10 ± 0.04
Glutamate	4.45 ± 0.001	6.76 ± 0.15	2.56 ± 0.1	2.32 ± 0.03	4.09 ± 0.11
Glycine	2.58 ± 0.001	3.87 ± 0.08	1.34 ± 0.04	1.62 ± 0.02	2.75 ± 0.14
Serine	1.73 ± 0.0003	2.72 ± 0.004	1.25 ± 0.06	1.30 ± 0.02	1.93 ± 0.05

NAN - *Nannochloropsis oceanica*; CHLO - *Chlorella vulgaris*; GRA - *Gracilaria gracilis*; ULVA - *Ulva rigida*; BLEND – blend of the four algae. ¹ AA, amino acids; ² Calculated by estimation: 100 – (crude protein + crude fat + ash). ³ EAA, essential amino acids; ⁴ CEAA, conditionally essential amino acids; ⁵ NEAA, nonessential amino acids.

Table 2. Single algae and commercial blend fatty acid composition (% total fatty acids and mg g⁻¹ DM).

	NAN	CHLO	GRA	ULVA	BLEND
Fatty acids (% total fatty acids)					
10:0	0.19 ± 0.04	-	-	-	-
12:0	0.60 ± 0.01	0.21 ± 0.01	-	-	0.54 ± 0.02
13:0	-	-	-	4.82 ± 0.08	-
14:0	6.02 ± 0.07	0.68 ± 0.04	23.02 ± 1.71	1.38 ± 0.04	5.35 ± 0.03
15:0	1.06 ± 0.02	1.73 ± 0.04	0.70 ± 0.02	3.05 ± 0.004	1.45 ± 0.03
16:0	26.58 ± 0.10	26.64 ± 0.09	39.61 ± 1.02	55.21 ± 0.39	30.54 ± 0.28
17:0	0.23 ± 0.02	0.82 ± 0.03	-	-	0.39 ± 0.02
18:0	0.35 ± 0.001	3.25 ± 0.001	0.71 ± 0.01	-	0.67 ± 0.05
21:0	-	-	-	8.15 ± 0.25	0.74 ± 0.02
22:0	-	-	-	2.02 ± 0.04	-
¹ ∑ SFA	35.02 ± 0.13	33.32 ± 0.11	64.05 ± 1.99	74.64 ± 0.47	39.57 ± 0.29
16:1	31.63 ± 0.37	2.90 ± 0.03	1.89 ± 0.04	11.04 ± 0.08	20.98 ± 0.38
17:1	0.23 ± 0.001	0.10 ± 0.02	-	0.66 ± 0.04	-
18:1 n-9 c (OA)	5.49 ± 0.04	5.33 ± 0.05	8.44 ± 0.19	1.52 ± 0.15	5.07 ± 0.11
² ∑ MUFA	37.84 ± 0.37	8.32 ± 0.06	10.33 ± 0.20	13.22 ± 0.18	26.04 ± 0.40
18:2 n-6 c (LA)	4.58 ± 0.02	23.56 ± 0.05	1.88 ± 0.18	3.43 ± 0.11	9.27 ± 0.04
18:3 n-3 (ALA)	0.63 ± 0.002	34.80 ± 0.26	-	8.72 ± 0.15	10.70 ± 0.20
20:3 n-6	0.30 ± 0.02	-	1.45 ± 0.06	-	0.43 ± 0.02
20:4 n-6 (ARA)	5.78 ± 0.07	-	22.29 ± 0.56	-	7.07 ± 0.16
20:5 n-3 (EPA)	16.34 ± 0.42	-	-	-	6.91 ± 0.41
³ ∑ PUFA	27.63 ± 0.43	58.36 ± 0.26	25.62 ± 0.59	12.15 ± 0.19	34.38 ± 0.49
n-3 PUFA	16.97 ± 0.42	34.80 ± 0.26	-	8.72 ± 0.15	17.61 ± 0.46
n-6 PUFA	10.66 ± 0.07	23.56 ± 0.05	25.62 ± 0.59	3.43 ± 0.11	16.77 ± 0.17
Fatty acids (mg g⁻¹ DM)					
¹ ∑ SFA	43.02 ± 0.25	26.93 ± 1.32	19.25 ± 0.86	17.55 ± 0.28	31.94 ± 1.88
² ∑ MUFA	45.14 ± 0.04	6.60 ± 0.30	3.01 ± 0.05	3.09 ± 0.07	20.73 ± 1.46
³ ∑ PUFA	31.84 ± 0.71	45.49 ± 1.73	7.20 ± 0.14	2.76 ± 0.10	26.36 ± 0.69
ARA	6.65 ± 0.15	-	6.25 ± 0.12	-	5.36 ± 0.23
EPA	18.68 ± 0.69	-	-	-	5.19 ± 0.04

NAN - *Nannochloropsis oceanica*; CHLO - *Chlorella vulgaris*; GRA - *Gracilaria gracilis*; ULVA - *Ulva rigida*; BLEND – blend of the four algae. ¹ ∑ SFA is the sum of saturated fatty acids; ² ∑ MUFA is the sum of monounsaturated fatty acids; ³ ∑ PUFA is the sum of polyunsaturated fatty acids.

2.3.2 Bactericidal and bacteriostatic activities of the algae

The bactericidal and bacteriostatic activities of the algae, single or blended, against some of the main groups of pathogenic bacteria for farmed fish are presented in Figure 1. Results showed that algae displayed bactericidal activity against many of the bacteria tested but were unable to kill *V. anguillarum* and *A. hydrophila*. The microalgae NAN and CHLO did not have bactericidal activity against *V. harveyi*, while, for this same bacterium, the macroalgae ULVA had the highest activity (18%) when compared to GRA (12%). Against *V. parahaemolyticus*, the two macroalgae and the blend showed the highest activity values (25-26%) when compared to NAN (13%). Regarding *Y. ruckeri*, once again ULVA displayed the highest capacity to kill this bacterium (25%) compared to the other algae but did not differ significantly from the BLEND (18%); the lowest bactericidal activity was observed for NAN (9%), followed by CHLO (11%) and GRA (16%). All algae displayed similar bactericidal activities (16-22% and 16-26%, respectively) against both *E. tarda* and *P. damsela* subsp. *piscicida* (Phdp). The highest bactericidal activity (40-45%) was observed against *T. maritimum*; all algae species and blend were equally effective, without significant differences among them (Figure 1a).

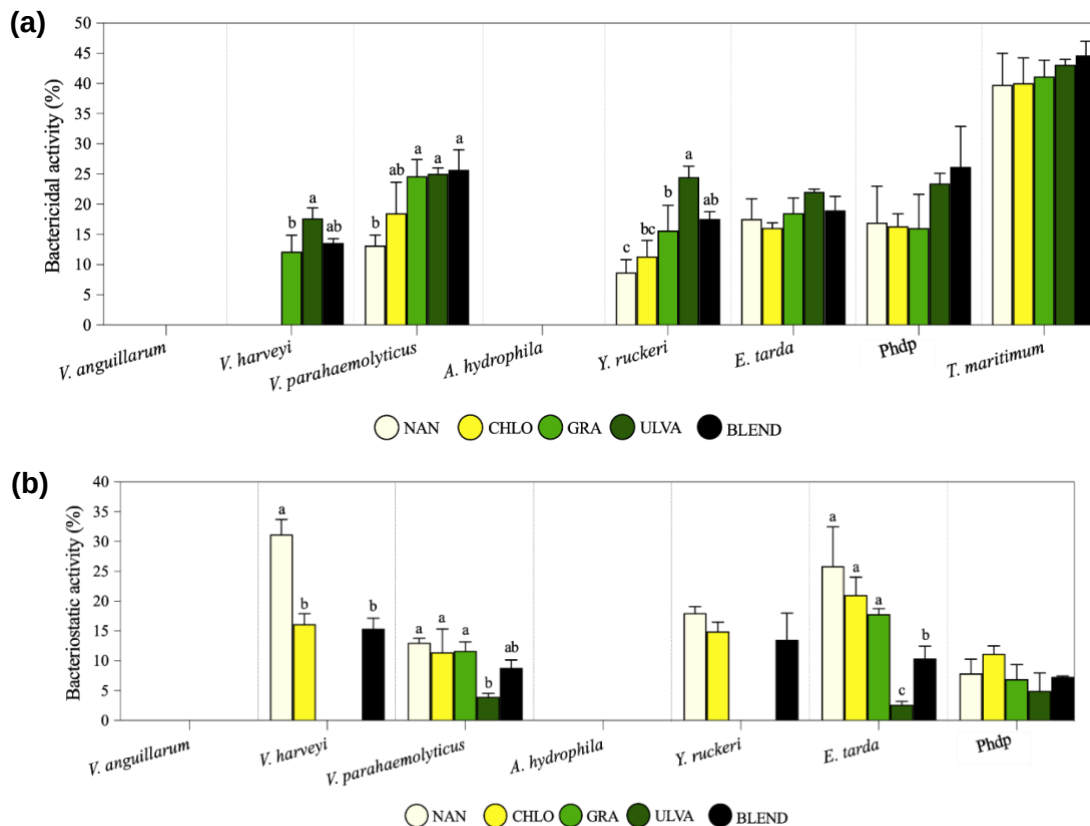


Figure 1. *In vitro* bactericidal (a) and bacteriostatic (b) activities of the tested algae against main pathogenic bacteria for farmed fish. NAN - *Nannochloropsis oceanica*; CHLO - *Chlorella vulgaris*; GRA - *Gracilaria gracilis*; ULVA - *Ulva rigida*; BLEND – blend of the four algae; Phdp -

Photobacterium damsela subsp. *piscicida*. Values are presented as means $\pm$ SD (n = 3 biological replicates) and different letters denote significant differences among treatments.

As observed for the bactericidal activity, the tested algae did not present any bacteriostatic activity against both *V. anguillarum* and *A. hydrophila*. Only the microalgae and the blend were able to inhibit *V. harveyi* growth, with NAN presenting higher bacteriostatic activity (31%) when compared with both CHLO (16%) and the BLEND (15%). Both microalgae and GRA displayed significantly higher activities against *V. parahaemolyticus* (11-13%) than ULVA (4%) but did not differ from the BLEND (9%). The microalgae and the blend showed similar ability to inhibit the growth of *Y. ruckeri* (14-18%), while no detectable activity was observed for the macroalgae. Concerning *E. tarda*, both microalgae and GRA had higher bacteriostatic activities (18-26%) compared to the blend (10%), and these activities differed from that of ULVA (3%) that presented the lowest capacity to inhibit the growth of this bacterium. All tested algae showed similar growth inhibition against Phdp (5-11%) (Figure 1b).

2.3.3 Antioxidant capacity of the algae

The antioxidant activity of each algae and the blend was evaluated by two spectrophotometric methods: ABTS⁺ and DPPH⁺ scavenging capacities. Concerning the ABTS assay, GRA displayed higher antioxidant capacity (14 $\mu\text{gTE mg}_{\text{DW}}^{-1}$) when compared to the other algae (10 $\mu\text{gTE mg}_{\text{DW}}^{-1}$ for NAN, CHLO and the BLEND). The macroalgae ULVA had the lowest antioxidant activity (6 $\mu\text{gTE mg}_{\text{DW}}^{-1}$). Based on the DPPH assay, GRA and the BLEND presented the highest antioxidant capacity (12-13 $\mu\text{gTE mg}_{\text{DW}}^{-1}$), that was significantly higher than that observed for NAN (7 $\mu\text{gTE mg}_{\text{DW}}^{-1}$) (Figure 2).

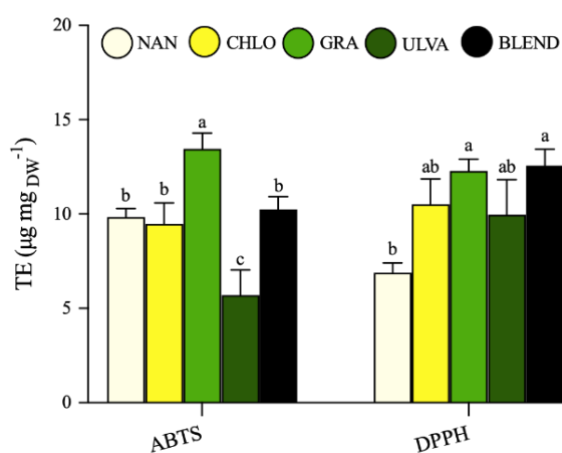


Figure 2. *In vitro* antioxidant capacity of the different algae. ABTS - 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid; DPPH - 1,1-diphenyl-2-picrylhydrazyl; NAN - *Nannochloropsis oceanica*; CHLO - *Chlorella vulgaris*; GRA - *Gracilaria gracilis*; ULVA - *Ulva rigida*; BLEND – blend of the four algae. Values are presented as means $\pm$ SD (n = 3 biological replicates) and expressed in trolox equivalents (TE, $\mu\text{g mg}_{\text{DW}}^{-1}$). Different letters denote significant differences among treatments.

2.4 Discussion

Aquaculture intensification can incur increased stress and disease outbreaks. Nutritional strategies to improve fish robustness and disease resistance can be a suitable solution and algae are added-valued natural products with high nutritional value and great potential to increase fish health status (Dawood, 2021). The nutritional value of algae varies greatly with harvesting season (macroalgae) and culturing conditions (microalgae and macroalgae). The composition of the selected microalgae *N. oceanica* and *C. vulgaris* (NAN and CHLO, respectively), and macroalgae *G. gracilis* and *U. rigida* (GRA and ULVA, respectively) was within the ranges reported earlier (Batista et al., 2020b; Neto et al., 2018; Valente et al., 2021). Both microalgae and the algae blend presented a relatively high protein content and can be considered good protein sources for aquafeeds. Nonetheless, the protein content of GRA was lower than previous batches commercially produced under integrated multi-trophic aquaculture conditions (Araújo et al., 2016; Batista et al., 2020b). According to Abreu et al. (2011) temperature and light are the main environmental factors that condition the growth and nutrient accrual ability of seaweeds with direct impact on their protein content. The true protein content of an ingredient is determined by the sum of the individual amino acids (AAs) (Mæhre et al., 2018), but most studies estimate crude protein indirectly, using the traditional nitrogen-to-protein conversion factor (Kp) of 6.25. Although large variations can be observed between- and within species, previous reports have suggested lower average Kp values (4.08-5.00) for microalgae and seaweeds, and indicated that a Kp of 6.25 overestimated protein content by 40% (Angell et al., 2016; Templeton & Laurens, 2015). But in the present study the algae protein contents estimated using a Kp of 6.25 were very close to true protein, calculated as the sum of AAs, with an overestimation of 5% for the seaweeds (GRA and ULVA), 2% for CHLO, and an underestimation of 1% for NAN and the BLEND. Different technical approaches related to ingredient processing prior protein extraction (e.g. hydrolysis time) and amino acid determination could partly explain such variable results among studies (Batista et al., 2020b; Biancarosa et al., 2017; Safi et al., 2013). The sum of total amino acids provides the most reliable indication of an algae protein content, and caution should be taken when selecting the nitrogen-to-protein factor to estimate total protein values, avoiding direct comparisons among studies using distinct factors. This is particularly relevant when the nutritional value of different algae species is being compared. Since the replacement of traditional marine feedstuffs by plant protein (PP) sources in aquafeeds can be associated with imbalances in essential amino acids (EAAs) (Gatlin III et al., 2007), the inclusion of algae rich in EAAs may be an advantageous tool in feed formulations. Our results indicated that the tested algae contained all EAAs although we observed variations among species. The microalgae CHLO is the best source of EAAs

(29%), especially lysine (7%) which is one of the most limiting EAAs in PP sources (Leinonen et al., 2019). Previous studies reported that FM contains 5% lysine and for PP (i.e., rice protein concentrate, soybean meal, rapeseed meal and cottonseed meal) the range is 2-3% (Cai et al., 2018; Mahmoud et al., 2020). Mahmoud et al. (2020) reported lysine levels of 5% DM in *C. vulgaris* with a protein content of 51%. Batista et al. (2020b) observed even lower values of lysine (4% DM) for a 54% protein level in this same microalga. Differences in culturing conditions or in methodologic approaches (e.g., digestion time prior to amino acid determination) could account for the observed differences. Nevertheless, CHLO appears as a rich source of lysine to be included in aquafeeds, overcoming the deficiencies associated with formulations with high levels of plant protein sources.

As in the case of protein, the lipid content and fatty acid composition in different species of algae is highly variable and depends on productions conditions (Francavilla et al., 2013; Pandit et al., 2017; Trigui et al., 2013; Xiao et al., 2013). NAN and the blend displayed a more balanced fatty acid composition (proportion of SFAs, MUFAs and PUFAs) compared to the other algae species, with the blend being able to retain a relatively high level of PUFAs (34%), the second highest level after CHLO (58%), comprising EPA (7%) provided by NAN and ARA (7%) from both NAN and GRA. It is of particular interest to produce an algae blend with high levels of PUFAs, particularly EPA and ARA, since these represent essential fatty acid for marine fish (Tocher, 2003). The tested blend had also moderate levels of SFAs (40%) compared with the high SFAs levels of the macroalgae (64 and 75%) and an intermediate level of MUFAs (26%). Overall, the microalgae *N. oceanica* and *C. vulgaris* are good sources of dietary protein (34-58%), lipids (6-7%) and EAA (17-29%).

Regarding the macroalgae (*G. gracilis* and *U. rigida*), they have higher carbohydrates content (46-53%). The capacity of fish to digest carbohydrates varies greatly with fish species, and high levels of carbohydrates are not advisable in feed formulations for carnivorous fish (Krogdahl et al., 2005). Nonetheless, the previously demonstrated prebiotic effect, antimicrobial activity and free-radical scavenging capacity of algal polysaccharides (Wijesekara et al., 2011) can be exploited by including carbohydrates-rich algae in functional aquafeeds to impart health benefits for farmed animals. From a nutritional point of view, the commercial blend (ALGAESSENCE™ – Feed, representing a well-balanced mix of the four algae) seems to be the most promising product to be included in aquafeeds, being a rich source of proteins (37%), lipids (5%), essential fatty acids (EPA and ARA, 7% each), essential amino acids (18%) and carbohydrates (34%).

Most studies on the *in vitro* antibacterial activity of algae are centred on specific compounds and the extracts used for the assays are prepared with organic solvents (e.g., ethanol, methanol, and acetone) and complex physical and/or chemical processes (Abu-Ghannam

& Rajauria, 2013; Ventura et al., 2017). The algae extract used in these *in vitro* studies were prepared in distilled water and at a low concentration (1 mg mL⁻¹), to better simulate the expected effects of algae when included without prior physical or chemical processing in aquafeeds (whole algae). Several compounds such as unsaturated fatty acids, carotenoids, and phenolic and polysaccharide components derived from micro- and macroalgae have been tested for their antibacterial activity (Kiran & Venkata Mohan, 2021; Silva et al., 2020). Although aqueous extracts were used in this study, most of the natural antimicrobial compounds are not water-soluble (Abu-Ghannam & Rajauria, 2013). Therefore, the antimicrobial activity observed herein is probably due to the action of water-soluble components present in algae such as some polysaccharides (Chojnacka & Kim, 2015). The cell walls of seaweeds are rich in water-soluble sulphated polysaccharides such as agar and ulvan that are present in *Gracilaria* sp. and *Ulva* sp., respectively (Sfriso et al., 2017; Wijesekara et al., 2011). Findings from a previous study indicated that soluble polysaccharides of *C. vulgaris* exhibit antimicrobial activity against both Gram-positive and Gram-negative bacteria (El-Naggar et al., 2020). On the other hand, another report pointed out the immunostimulatory potential of water-soluble polysaccharides from *Nannochloropsis* sp. (Pandeirada et al., 2019). It is necessary to perform a better chemical characterisation of each extract in order to identify the bioactivities of the main compounds. The algae were able to kill and inhibit the growth of most of the pathogenic bacteria tested, excepted for *V. anguillarum* and *A. hydrophila*. The latter one is a serious pathogen that affects mostly freshwater fish, while *V. anguillarum* is the causative agent of vibriosis, one of the most common diseases among farmed fish and shellfish. Although immunization by vaccination has been implemented against *V. anguillarum*, this deadly disease is still responsible for heavy economic losses in aquaculture, and in some situations, the use of antibiotics is still required (Toranzo et al., 2017). In order to reduce the use for antibiotics and limit antibiotics resistance, new approaches, including the use of pre- and probiotics, have been tested to prevent and control disease outbreaks (Rengpipat et al., 2008; Ringø et al., 2010). But further efforts should be directed towards finding novel strategies to improve fish robustness and reduce the costs associated with disease management. Our observations indicated the bactericidal activity of macroalgae and bacteriostatic activity of microalgae against *V. harveyi*. Furthermore, the macroalgae did not display bacteriostatic activity against *Y. ruckeri*. These results indicate that micro- and macroalgae have different antibacterial mechanisms of action against these bacteria (stasis or cidal activity, respectively). Nevertheless, the combination of the blend was able to both kill and inhibit the growth of these pathogens. Therefore, the BLEND seems to be a promising ingredient to be included in aquafeeds; an added-value component when compared to the inclusion of a single algae. In general, the macroalgae (specifically ULVA) and the BLEND presented

the highest bactericidal activity against the tested pathogens, while the microalgae displayed a higher bacteriostatic activity. The most promising results were observed against *T. maritimum*, with all algae performing equally well and producing a 40-45% activity. *T. maritimum* causes tenacibaculosis that is manifested as skin and gill lesions; the disease is currently widely spread in Europe and poses a threat to the culture of a wide range of marine fish species (Toranzo et al., 2005). A vaccine against *T. maritimum* is currently available for some farmed fish species but not for all (Miccoli et al., 2019). So, the use of key ingredients in feeds, capable of improving fish resistance and avoiding high mortalities during a bacterial infection, is particularly pertinent for the growth of the aquaculture sector. Such a feeding strategy would be particularly relevant in hatcheries since larvae do not have a fully developed immune system and thus vaccination is not feasible until a later stage. In this context, the *in vitro* antibacterial activity displayed by the selected algae, especially the blend, against a wide range of pathogens affecting farmed species demonstrate the potential of these marine products to be included as health additives in feeds for the early stages of fish to improve their robustness.

Algae are rich sources of bioactive compounds with antioxidant activity (e.g., carotenoids, certain polysaccharides, and polyphenols), and the scavenging properties of different algae has been vastly explored in the past years (Gomez-Zavaglia et al., 2019; Sansone & Brunet, 2019). In the present study, individual algae and the blend presented a certain degree of antioxidant activity. Similar to what was observed for the antibacterial activity, the antioxidant capacity of the extracts could be attributed to the presence of water-soluble components (e.g., algal polysaccharides), rather than carotenoids that require different extraction methods and application of solvents. The ABTS scavenging activity of the water extracts was higher in GRA (13.5 $\mu\text{gTE mg}_{\text{DW}}^{-1}$) and lower in ULVA (5.7 $\mu\text{gTE mg}_{\text{DW}}^{-1}$). Both GRA and the BLEND had the highest DPPH activity (12.3 and 12.6 $\mu\text{gTE mg}_{\text{DW}}^{-1}$, respectively) and NAN (DPPH, 6.9 $\mu\text{gTE mg}_{\text{DW}}^{-1}$) the lowest. These results suggest that the macroalga GRA used in this study either had a higher number of water-soluble antioxidant compounds or these compounds were more easily available in the aqueous solution. The high carbohydrate content (51%) of this algal extract, particularly the water-soluble polysaccharide agar, may, at least partially, contribute to the observed higher antioxidant activity. The comparison with results from the literature is difficult since the units used for expressing the activity vary greatly. Nevertheless, a previous study on *G. gracilis* reported a wide range of ABTS scavenging activities (5 to 108 $\mu\text{gTE mg}_{\text{DW}}^{-1}$); the differences can be related to harvesting season and different solvents used in several steps of the extraction process (Francavilla et al., 2013). In the present study, the samples that were prepared in a single extraction step of the algae in water, had a considerable antioxidant activity.

Overall, the nutritional characterisation and *in vitro* studies showcased the commercial micro- and macroalgae blend, compared to individual algae, as a promising ingredient to impart added value to aquafeeds. Moreover, the use of such functional feeds could promote both fish growth and health while being sustainable at social, economic, and environmental levels and contributing to the one health concept. But further studies are still warranted to ascertain the full potential of these products in their future *in vitro* and *in vivo* applications.

2.5 Author's contributions

Conceptualization, Benjamin Costas and Luisa M. P. Valente; methodology, Mariana Ferreira and Carla Teixeira; validation, Benjamin Costas and Luisa M. P. Valente; formal analysis, Mariana Ferreira; investigation, Mariana Ferreira and Carla Teixeira; resources, Helena Abreu and Joana Silva; data curation, Mariana Ferreira; writing - original draft preparation, Mariana Ferreira; writing - review and editing, Carla Teixeira, Helena Abreu, Joana Silva, Benjamin Costas, Viswanath Kiron and Luisa M. P. Valente; supervision, Benjamin Costas, Viswanath Kiron and Luisa M. P. Valente; funding acquisition, Benjamin Costas and Luisa M. P. Valente. All authors have read and agreed to the published version of the manuscript.

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2.7 Data availability

Data will be made available on reasonable request.

2.8 Conflict of interest

The authors declare no conflict of interest.

Chapter 3.

***Gracilaria gracilis* and *Nannochloropsis oceanica*, singly or in combination, in diets alter the intestinal microbiota of European seabass (*Dicentrarchus labrax*)**

By

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Abstract

Algae feeds and fish gut microbiota have been given importance in the past few years because of the necessity to rely on sustainable ingredients in aquafeeds and the link of host-associated microbes to organismal health. But little is known about the potential of algae, particularly of micro- and macroalgae combination, to shape the intestinal bacterial communities. Hence, in the present work, the 16S rRNA gene sequencing was employed to unravel the effects of the seaweed *Gracilaria gracilis* and the microalga *Nannochloropsis oceanica* - included either singly or in combination in the diets of European seabass - on the diversities and composition of the gut bacterial communities. Results indicated that 8% inclusion of either *G. gracilis* (GRA) or *N. oceanica* (NAN) led to a reduction in the gut microbial diversity. On the other hand, inclusion of the micro- and macroalga in a blend (NANGRA) mitigated these plausible effects on the intestinal bacterial communities. The core microbiota of European seabass was composed of both beneficial (*Lactobacillus* and *Cetobacterium*) and potentially pathogenic (*Flavobacterium*) bacteria. The GRA diet was associated with a lower abundance of carbohydrate degraders, while also promoting the growth of bacteria capable of outcompeting fish pathogens (*Sulfitobacter* and *Methylobacterium*); while the NAN diet led to a higher representation of the genus *Bacillus*, with probiotic potential, accompanied by a decrease in *Vibrio*, a genus encompassing several fish pathogenic species. These findings demonstrate the ability of micro- and macroalgae to modulate the gut microbiota of European seabass, with plausible implications to host gut homeostasis.

Keywords: seaweed, microalga, algae blend, European seabass, intestinal microbiota

3.1 Introduction

Fish intestinal microbiota has become one of the most studied topics in the field of aquaculture, by following the trends in human and mammalian research (Diwan et al., 2022). The complex and dynamic assemblage of microorganisms in the gut microbiota is known to have crucial implications on key physiological functions (Cerf-Bensussan & Gaboriau-Routhiau, 2010; Egerton et al., 2018). Since the intestinal bacterial communities of aquatic animals are vastly shaped by diet, it is expected that nutritional manipulation of fish gut microbiota using added-value products can produce intended benefits on fish welfare and nutrition (Yukgehnaish et al., 2020). In this context, it is worth mentioning that algae are natural products and are rich in biologically active substances capable of promoting fish growth and improving the immune status and disease resistance (Valente et al., 2021; Wan et al., 2019). Specifically, two algae that have been recently explored by the feed industry are the macroalga *Gracilaria gracilis* and the microalga *Nannochloropsis oceanica*. Although there are some reports on the effect of these algae on fish growth performance, immunity, oxidative status and intestine histomorphology (Batista et al., 2020a; Passos et al., 2021; Sørensen et al., 2021), little is known about their impact on the intestinal microbiota of farmed fish.

The core research on dietary modulation of fish gut microbiota has been undertaken to reveal the effect of either probiotics and prebiotics or dietary proteins and lipids (Egerton et al., 2018; Pérez-Pascual et al., 2020; Vargas-Albores et al., 2021). Studies that evaluated the changes in intestinal microbiota of farmed fish fed algae-supplemented diets have also emerged more recently (Keating et al., 2021; Sagaram et al., 2021). These studies focused on the impact of algae as feed ingredients (replaced fish meal/fish oil) (Li et al., 2022; Lyons et al., 2017; Rico et al., 2016) or feed additives (Cerezo et al., 2022; Jorge et al., 2019). Replacing fish meal (partially) with the macroalgae *G. cornea* or *Ulva rigida* altered the diversity of bacteria in gilthead seabream (*Sparus aurata*): while 15% substitution of both the seaweeds increased the diversity, 25% substitution of *U. rigida* decreased it (Rico et al., 2016). Li et al. (2022) reported the effects of total fish meal replacement with the microalga *Chlorella vulgaris* on the intestinal microbiota of largemouth bass (*Micropterus salmoides*); the abundance of beneficial taxa (e.g., *Cetobacterium* that are known as vitamin B₁₂ producers) was increased without any differences in alpha diversity compared to the fish meal group. On the other hand, in rainbow trout (*Oncorhynchus mykiss*), 5% dietary *Schizochytrium limacinum* as partial fish oil substitute significantly increased the intestinal bacterial diversity without changing the overall microbial community structure. Nevertheless, some microbial groups including members of the lactic acid bacteria, which are commonly considered beneficial, had an increased representation in the *S. limacinum*

group (Lyons et al., 2017). Short-term feeding of gilthead seabream with diets supplemented with *N. gaditana* at 7.5 g kg⁻¹ led to an increased richness of the gut microbiota, but not significant when compared with fish fed the control diet (Jorge et al., 2019). Similarly, the diversity of gut microbiota was not altered in Atlantic cod (*Gadus morhua*) fed diets supplemented with *U. rigida* (10%) compared to the control group (Keating et al., 2021).

Based on the results reported in the available literature, micro- and macroalgae, either as ingredients or supplements, seem to modulate the gut microbiota of farmed fish, but the effects are species-specific and vastly dependent on the type of the algae and their inclusion levels. Moreover, most studies have reported the effects of individual algae. A mix of micro- and macroalgae in aquafeeds can impart synergistic modulatory effects on the gut microbiota. However, this topic is underexplored and deserves more attention because both micro- and macroalgae can be future feed ingredients/additives. Here we report for the first time the impact of the seaweed *G. gracilis* and the microalga *N. oceanica*, incorporated singly or in combination, on the composition of the microbial communities in the posterior intestine of European seabass (*Dicentrarchus labrax*), which is one of the most important farmed fish species of the Mediterranean region.

3.2 Materials and methods

3.2.1 Ethical statement

The trial was performed by accredited scientists, and the animal handling and sampling procedures were in compliance with the guidelines of the European Union (directive 2010/63/EU) and Portuguese law (Decreto-Lei no. 113/2013, de 7 de Agosto) on the protection of animals used for scientific purposes. Review of the ethical process concerning all animal handling and sampling procedures was performed by CIIMAR animal welfare body (ORBEA-CIIMAR) and approved by national competent authorities.

3.2.2 Experimental diets, feeding trial and sampling

Details of the experimental design and diets for the feeding trial, as well as zootechnical data can be found in Batista et al. (2020a). Briefly, European seabass juveniles (approximately 6 months of age) from a commercial fish farm (SONRIONANSA S.L., Cantabria, Spain) were transported to CIIMAR facilities in Matosinhos, Portugal. Following a 2-week quarantine, twelve homogeneous groups (average body weight of 29.7 ± 0.02 g and total length of 13.7 ± 0.08 cm) of nineteen fish were transferred into 50-L fiberglass tanks of a saltwater recirculation system (RAS, density of 11.3 kg m^{-3}). The RAS conditions

were as follows: water temperature of 21 °C, salinity of 35‰, flow rate at 4 L min⁻¹ and 12 h light/12 h dark photoperiod regime.

The experimental diets consisted of four isoproteic (53% dry matter, DM) and isolipidic (17% DM) diets supplied by Sparos Lda.: a commercial feed-based diet (CTRL) and three experimental diets with either 8% *G. gracilis* (GRA) or 8% *N. oceanica* (NAN) or a blend of 4% of each alga (NANGRA), included at the expense of fish meal and wheat meal. Ingredients and proximate composition of the experimental diets are summarized in Figure 1. To unravel the impact of the macroalga *G. gracilis* and the microalga *N. oceanica*, added singly or in combination in diets, on European seabass intestinal microbiota, fish were fed the experimental diets for 106 days. Fish in triplicate tanks per dietary treatment were fed three times a day until apparent satiation. The biotic conditions (e.g., temperature and salinity), known to have a greater impact on the gut microbiota, were kept constant throughout the feeding trial.

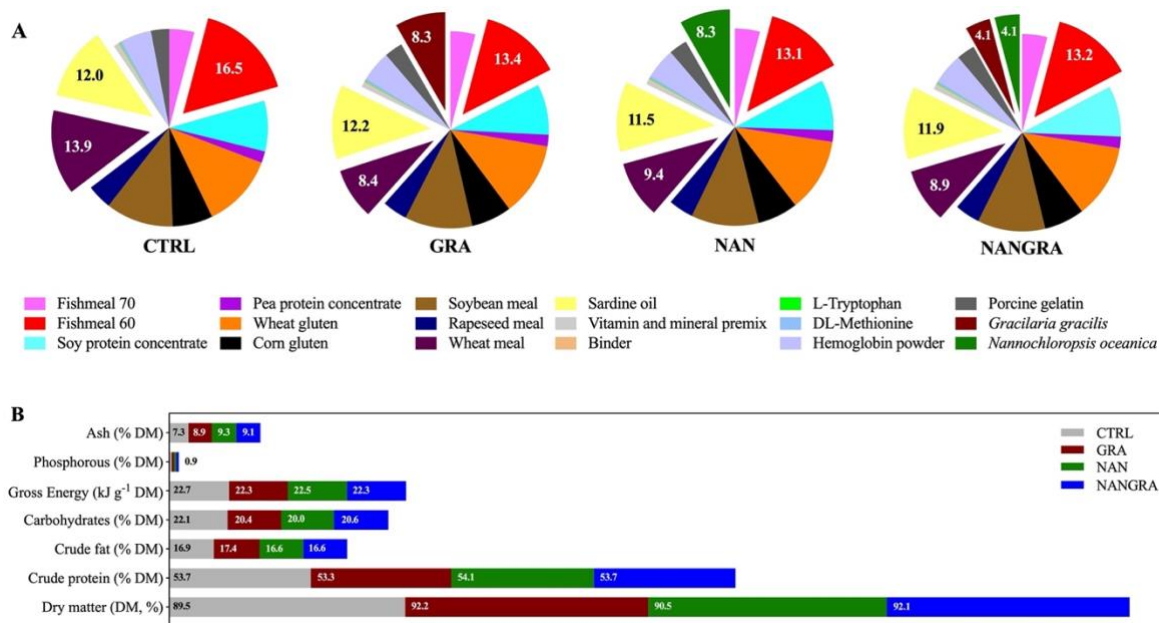


Figure 1. Ingredients in % (A) and proximate composition in % DM (B) of the experimental diets. Adapted from Batista et al. (2020a).

At the end of the 15-week feeding trial, fish were starved for 24 h and sacrificed by a sharp blow on the head before sampling the tissues. The gastrointestinal tract of four fish per tank (12 fish per treatment) was removed under sterile conditions and the posterior intestine (section preceding the ileorectal valve) was opened using sterile scissors. A sterile swab (Copan Italia, Italy) was used to collect the mucus from the posterior portion. The swab was then placed in a sterile tube and immediately frozen in liquid nitrogen. Samples were stored at -80 °C until further analysis.

3.2.3 DNA extraction for 16S rRNA sequencing

All the procedures mentioned here were performed under sterile conditions. For DNA extraction, three samples per treatment were randomly selected to divide samples into 4 batches. Genomic DNA was extracted from all samples using QIAamp DNA stool Mini Kit (Qiagen, Germany) according to the manufacturer's protocol, with some modifications. Briefly, swabs with mucus from the posterior intestine were first transferred to 5 mL tubes containing 1.4- and 0.1-mm Zirconium oxide beads (Cayman chemical, USA) and 2 mL of InhibitEX buffer (Qiagen). Afterwards, samples were homogenized using Precellys Evolution tissue homogenizer at 6,000 RPM (3 cycles of 30 s, with a 30 s pause in-between) following centrifugation for 5 min at 12,000 *g*. The obtained pellet was then resuspended, transferred to new 2 mL tubes, and incubated at 70 °C for 20 min. Following incubation, samples were mixed with a vortex for 1 min and centrifuged for 1 min at 12,000 *g*. The supernatant (600 µL) was transferred into a 2 mL tube with 25 µL of proteinase K, and the following steps of the DNA extraction proceeded according to the manufacturer's protocol (QIAamp DNA stool Mini Kit). Finally, DNA was eluted using 75 µL of ATE buffer and DNA quantity was checked using Qubit™ dsDNA Assay (Thermo Fisher Scientific Inc., USA).

3.2.4 Amplicon library preparation and sequencing

Amplicon libraries were prepared under sterile conditions. The first PCR reaction targeted the hypervariable V3-V4 region of the 16S rRNA gene, using specific bacterial primers 341F (5' CCTACGGGNGGCWGCAG 3') and 805R (5' GACTACNVGGGTWTCTAATCC 3') (Klindworth et al., 2013) flanked by Illumina adapters (~ 460 bp; Illumina, USA). The PCR reactions were performed in triplicate for each sample in a 25 µL final volume, with 1 µL of each primer (10 µM), 2.5 µL of DNA template (5 ng/µL), 8 µL of water, and using 12.5 µL of Amplitaq Gold Q5 (Thermo Fisher Scientific Inc.). Thermal cycling conditions were as follows: initial denaturation step at 95 °C for 10 min, 37 cycles at 95 °C for 30 s, 57 °C for 30 s, 72 °C for 1 min, and the final extension step at 72 °C for 7 min. Negative PCR controls, without DNA template, were also included. At the end of the amplification, the replicates for each sample were pooled together for the subsequent procedures. The amplified products were visualized on an agarose gel (1.5%), and the CleanNGS system (CleanNA, Netherlands) was used to purify the PCR products, according to the manufacturer's recommendations.

A second PCR (index PCR) was performed using the purified products, using Nextera XT Index primers (Illumina) and with the following thermal cycling conditions: 95 °C for 3 min, 8 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 min, and a final step at 72 °C for 5 min (16S Metagenomic Sequencing Library Preparation, Illumina). The CleanNGS system

(CleanNA) was then used to purify the amplicon libraries. Quality of the obtained libraries was evaluated on a Tapestation 2200 platform (Agilent Technologies, USA), and the libraries were subsequently quantified using the Quant-IT PicoGreen dsDNA assay kit (Thermo Fisher Scientific Inc.) and the Synergy2 microplate reader (Biotek, USA). Thereafter, the pooled library was quantified by Real-Time qPCR LightCycler 480 (Roche, Switzerland), using the KAPA Library quantification kit (Roche). The libraries were sequenced on an Illumina MiSeq (PE300) platform (MiSeq Control Software 2.5.0.5 and Real-Time Analysis software 1.18.54.0).

3.2.5 Sequence data analysis

Raw sequence data is deposited in the Sequence Read Archive (SRA) and the accession number is PRJNA867546. The obtained reads were first truncated at 270 bp by VSEARCH (Rognes et al., 2016). Next, MICCA pipeline (v1.7.2) (Albanese et al., 2015) was used to further process the reads. Employing MICCA, the sequences were merged at a minimum overlap length of 60 bp and a maximum mismatch of 20 bp. For further analysis, reads without primers were discarded and the forward and reverse primers were trimmed off from the merged reads. Next, sequences with an expected error rate of > 0.75 (Edgar & Flyvbjerg, 2015) were filtered and sequences shorter than 400 bp were removed. The “de novo UNOISE” method implemented in MICCA was employed to perform denoising on the filtered reads. The MICCA pipeline uses UNOISE3 algorithm (Edgar, 2016) that corrects sequencing errors and determines true biological sequences at single-nucleotide resolution. Chimera and mitochondrial sequences were removed, and the amplicon sequence variants (ASVs) were generated for the downstream analyses. The RDP classifier was used to assign taxonomies to the bacterial ASVs. The alignment of the sequences was then performed using the NAST multiple sequence aligner (DeSantis et al., 2006), and a phylogenetic tree was generated using the FastTree software within the MICCA pipeline.

3.2.6 Phylogenetic analysis

Blastn (Chen et al., 2015) of the Basic Local Alignment Search Tool (BLAST) was employed to investigate the similarities of the sequences of the identified ASVs and bacterial sequences from the NCBI Reference Sequence Database. Bacterial sequences with 100% match/similarity were selected for the phylogenetic analysis. Thereafter, these sequences were imported into Molecular Evolutionary Genetics Analysis (MEGAX) software (Kumar et al., 2018) and multiple sequence alignment of the ASVs and sequences with high hits in BLASTn was performed using ClustalW aligner (Version 2.0) (Larkin et al., 2007). Phylogenetic analysis of the sequences was performed using the unweighted average

binding among clusters (Unweighted Pair Group Method with Arithmetic mean, UPGMA) approach (Rédei (Ed.), 2008), by applying default parameters and 500 bootstrap replicates. The generated phylogenetic trees (using maximum likelihood inference) were visualized using MEGAX software.

3.2.7 Statistical analysis

All statistical analysis were performed on R studio version 1.4.1103. The packages “iNEXT” and “phyloseq” were used to calculate alpha diversity: overall species richness (function “ChaoRichness”), Shannon diversity (function “ChaoShannon”) and Simpson diversity (function “ChaoSimpson”). Faith’s phylogenetic diversity was calculated using the function “pd” in package “picante” to reveal the losses and gains in species. Kruskal-Wallis’ test followed by Dunn’s test were employed to understand the differences between the experimental groups. Then employing the functions in “ggplot2”, plots were generated for overall species richness, Shannon diversity, Simpson diversity and Faith’s phylogenetic diversity (Hsieh et al., 2016). For the bacterial beta diversity analysis, weighted UniFrac distances were employed (Lozupone & Knight, 2005), and beta diversity was visualized on a principal coordinates analysis (PCoA) plot. Dispersions of the communities were first analyzed using “betadisper” and significant dissimilarities between the communities were then determined using Permutational Multivariate Analysis of Variance Using Distance Matrices (PERMANOVA with 9,999 permutations) (Anderson, 2001), implemented in “adonis” function of the vegan R-package (Oksanen et al., 2013), followed by pairwise comparisons. The package “microbiome” was employed to determine relative abundance of core taxa (Lahti et al., 2017). To identify the differentially abundant ASVs, the non-rarefied data (McMurdie & Holmes, 2014) was analyzed using the “DESeq2” package (Love et al., 2014) as rarefied data is reported to reduce the statistical power of the analysis (Weiss et al., 2017).

3.3 Results

3.3.1 Composition of the intestinal microbiota of European seabass fed the algae diets

All samples were sequenced in a single MiSeq run, generating a total of 4,674,383 high-quality reads with an average of 101,617 reads per sample. To account for the read count variation in the different samples, the reads were rarefied to 18,000 sequences per sample; to obtain a uniform sampling depth for comparing the different groups. Out of the 45 samples, two libraries with several reads below the cut off were discarded. A total of 43 samples were used for the downstream analysis – 11 samples for the CTRL group, 11

samples for the GRA group, 12 samples for the NAN group and 9 samples for the NANGRA group. Across all samples, 4371 ASVs were identified after denoising, belonging to 26 phyla and 306 genera.

Concerning the abundances of the intestinal bacteria of European seabass, the 26 bacterial phyla identified across all samples are displayed in Figure 2A (relative abundance per sample) and Supplementary Figure 1A (abundance per dietary treatment). The most abundant phyla were Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, Fusobacteria, Nitrospirae and Parcubacteria (Figure 2B). Across all experimental groups, Proteobacteria was the most abundant phylum, with an average relative abundance of 35.25% in the CTRL group, 53.29% in the GRA group, 34.93% in the NAN group and 40.45% in the NANGRA group. The second most abundant phylum for the CTRL group was Bacteroidetes (12.06%), Actinobacteria for the GRA group (11.83%), and Firmicutes for the NAN and NANGRA groups (20.98 and 14.66%, respectively). The third most abundant phyla were Nitrospirae for the CTRL group (10.21%), Bacteroidetes for both GRA and NANGRA groups (9.61 and 11.46%, respectively), and Actinobacteria for the NAN group (12.65%) (Table 1).

The abundance of the dominant genera per dietary treatment are presented in Supplementary Figure 1B. The dominant genera included *Acinetobacter*, *Cetobacterium*, *Corynebacterium*, *Enhydrobacter*, *Flavobacterium*, *Micrococcus*, *Nitrospira*, *Parcubacteria*, *Prevotella*, *Pseudoalteromonas*, *Sphingomonas* and *Streptococcus*, but we did not find a clear dominance of specific genera across all intestinal samples (Figure 2C). The proportions of the dominant genera varied according to dietary treatment: the most abundant genus for the CTRL group was *Nitrospira* (10.03%); for the GRA group the most abundant taxa were *Pseudoalteromonas* (7.57%) and *Acinetobacter* (7.02%); for the NAN group *Streptococcus* had the highest proportion (6.54%); and for the NANGRA group *Acinetobacter* had a higher representation (8.23%) (Table 1).

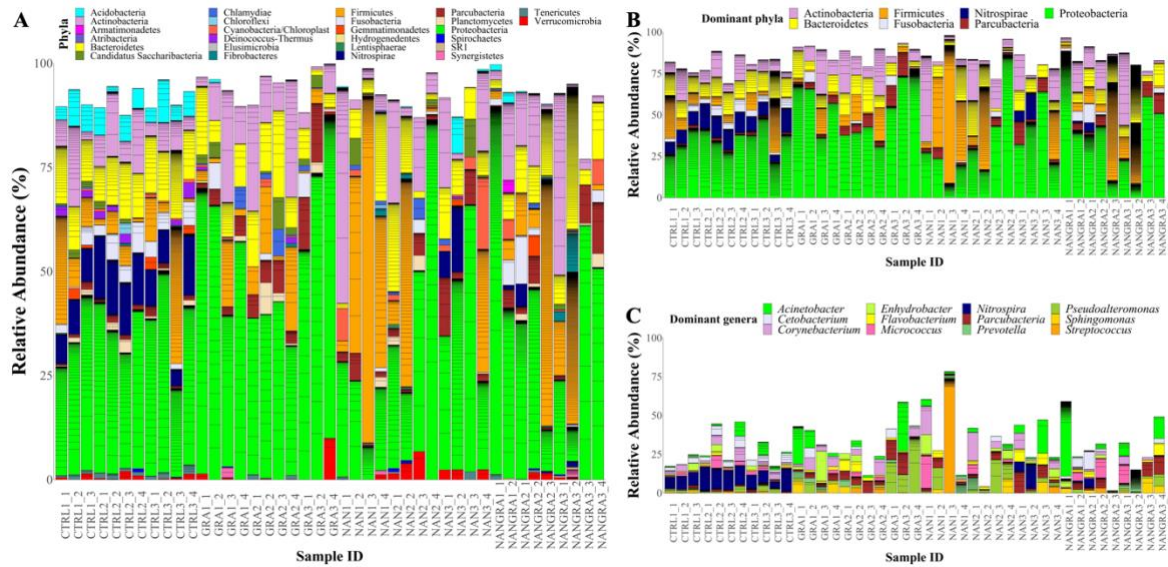


Figure 2. Relative abundance of the bacterial phyla (A), dominant phyla (B) and dominant genera (C) in the posterior intestine of European seabass fed the experimental diets. Sample ID: CTRL, control group; GRA, *Gracilaria gracilis* group; NAN, *Nannochloropsis oceanica* group; NANGRA, *G. gracilis* and *N. oceanica* blend group. Color codes: Actinobacteria – pink, Bacteroidetes – yellow, Firmicutes – orange, Fusobacteria – lavender, Nitrospirae – dark blue, Parcubacteria – dark red, Proteobacteria – green. Unclassified bacteria are not shown in the figures.

Table 1. Average relative abundance (%) of the dominant bacterial phyla and genera in the posterior intestine of European seabass fed the experimental diets.

	CTRL	GRA	NAN	NANGRA
Phyla				
Actinobacteria	8.34 ± 5.81	11.83 ± 10.11	12.65 ± 15.32	11.16 ± 14.19
Bacteroidetes	12.06 ± 2.25	9.61 ± 5.77	8.79 ± 7.09	11.46 ± 9.63
Firmicutes	8.99 ± 10.66	6.30 ± 7.39	20.98 ± 27.31	14.66 ± 20.10
Fusobacteria	3.84 ± 2.14	1.31 ± 2.41	0.48 ± 0.95	2.75 ± 3.86
Nitrospirae	10.21 ± 3.56	0.002 ± 0.01	1.85 ± 4.79	0.71 ± 1.79
Parcubacteria	1.27 ± 0.60	5.55 ± 4.43	3.79 ± 4.09	4.00 ± 4.45
Proteobacteria	35.25 ± 7.93	53.29 ± 16.57	34.93 ± 22.14	40.45 ± 25.35
Genera				
<i>Acinetobacter</i>	2.27 ± 3.23	7.02 ± 10.92	3.49 ± 5.89	8.23 ± 15.71
<i>Cetobacterium</i>	3.76 ± 2.13	1.31 ± 2.41	0.42 ± 0.97	2.70 ± 3.90
<i>Corynebacterium</i>	2.88 ± 2.79	3.64 ± 3.66	4.95 ± 7.02	1.34 ± 1.75
<i>Enhydrobacter</i>	1.21 ± 1.78	2.64 ± 5.73	2.25 ± 3.82	0.86 ± 1.86
<i>Flavobacterium</i>	0.93 ± 0.70	2.19 ± 2.45	2.39 ± 2.82	1.84 ± 2.57
<i>Micrococcus</i>	2.02 ± 2.24	0.72 ± 1.20	2.47 ± 6.20	3.48 ± 5.21
<i>Nitrospira</i>	10.03 ± 3.36	0.002 ± 0.01	1.77 ± 4.52	0.71 ± 1.79
<i>Parcubacteria</i>	1.27 ± 0.60	5.55 ± 4.43	3.79 ± 4.09	4.00 ± 4.45
<i>Prevotella</i>	0.33 ± 0.51	1.40 ± 1.77	1.25 ± 2.65	1.75 ± 4.40
<i>Pseudoalteromonas</i>	1.26 ± 1.49	7.57 ± 11.18	4.73 ± 6.70	1.63 ± 2.39
<i>Sphingomonas</i>	0.29 ± 0.69	1.60 ± 2.86	1.56 ± 3.18	2.09 ± 3.25
<i>Streptococcus</i>	0.22 ± 0.39	0.75 ± 1.41	6.54 ± 21.20	0.48 ± 1.08

Values presented as mean ± SD.

3.3.2 Impact of the algae diets on the diversity of the intestinal microbiota

The alpha diversity analysis, displayed in Figure 3A, was evaluated using the ecological diversity measures Chao richness (measure of species richness), Shannon diversity (effective number of common species that indicates the even distribution of microbes) and Simpson diversity (effective number of dominant species). The overall species richness was significantly lower in fish that consumed the GRA diet compared to CTRL ($P = 0.003$), but not compared to fish fed the NAN ($P = 0.06$) and NANGRA ($P = 0.36$) diets (Figure 3A1; Kruskal-Wallis' chi-squared = 13.467; $P = 0.0037$). The microbial evenness of the populations (Shannon diversity) in single algae groups (GRA and NAN) was significantly lower than in CTRL-fed fish ($P = 0.009$), but a similar difference was not observed for fish that consumed the algae blend (NANGRA; $P = 0.14$) (Figure 3A2; Kruskal-Wallis' chi-squared = 12.593; $P = 0.0056$). Similarly, fish that consumed both GRA and NAN diets presented a significant reduction in the effective number of dominant species (Simpson diversity) compared to the CTRL fish ($P = 0.02$ and $P = 0.01$, respectively), while such differences were not observed in NANGRA fish ($P = 0.15$) (Figure 3A3; Kruskal-Wallis' chi-squared = 11.442; $P = 0.0096$).

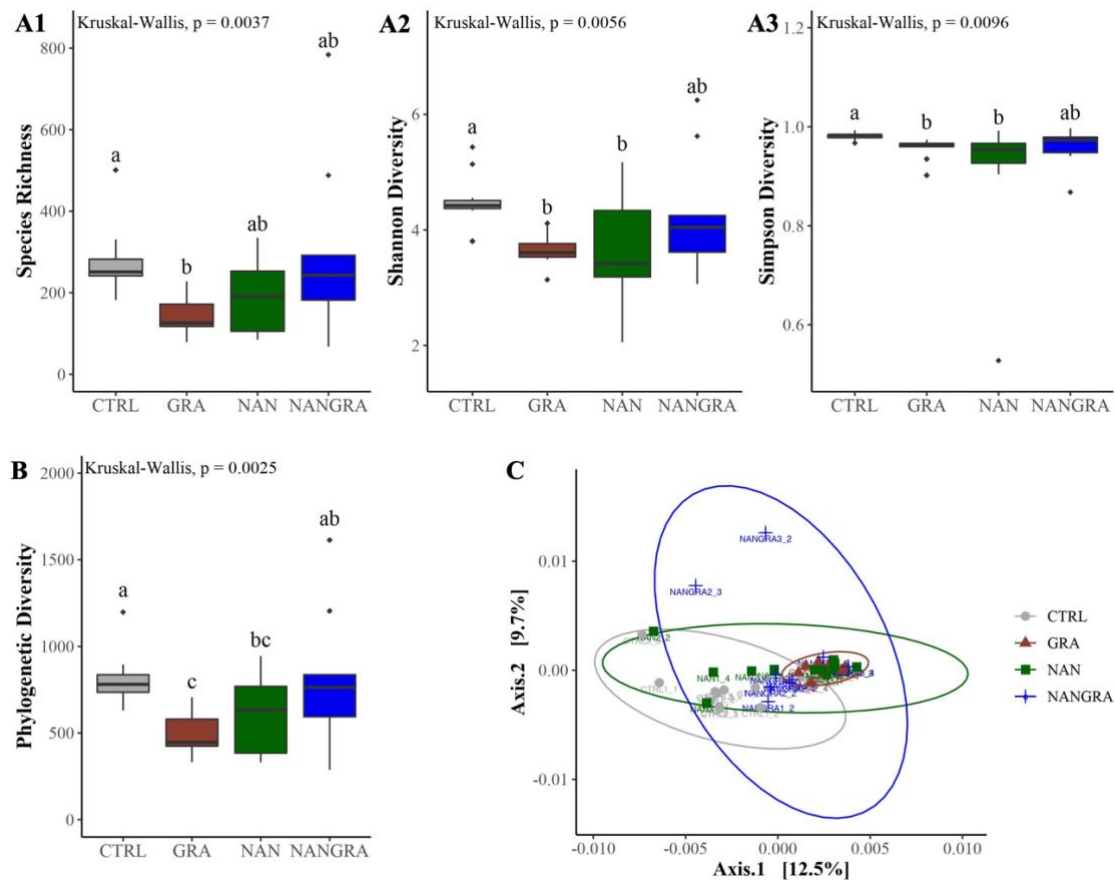


Figure 3. Diversity of the bacterial communities of the posterior intestine of European seabass fed the experimental diets. (A) Alpha diversity: species richness - A1, Shannon diversity - A2, Simpson

diversity - A3; (B) Faith's phylogenetic diversity; (C) Beta diversity visualized by a principal coordinate analysis (PCoA) plot and employing weighted UniFrac distances. Different letters denote statistically significant differences ($P < 0.05$) between the dietary treatments (CTRL, control group; GRA, *Gracilaria gracilis* group; NAN, *Nannochloropsis oceanica* group; NANGRA, *G. gracilis* and *N. oceanica* blend group).

Faith's phylogenetic diversity was also lower in fish fed the GRA ($P = 0.002$) and NAN ($P = 0.04$) diets compared to CTRL, but once again not in fish that consumed the blend diet ($P = 0.29$). Fish fed the GRA diet had also a significantly lower Faith's phylogenetic diversity compared to the NANGRA fish ($P = 0.04$) (Figure 3B; Kruskal-Wallis' chi-squared = 13.323; $P = 0.0025$). For the beta diversity analysis, we incorporated weighted UniFrac distance metric to understand the dissimilarities of the community structure. The principal coordinates analysis (PCoA) plot indicated that the first two components explained 12.5 and 9.7% of the variance within the dataset. Results from the PERMANOVA test revealed that the bacterial communities of fish fed the GRA diet were significantly different from those of the CTRL fish ($P = 0.01$) (Figure 3C; F-statistic = 1.932, $R^2 = 0.129$, $P = 0.001$).

3.3.3 Core bacterial taxa of the intestinal microbiota of European seabass

For the core microbial analysis, a prevalence and detection thresholds of 90 and 0.2%, respectively, were concomitantly applied. Results indicated that the core microbial taxa of the posterior intestine of European seabass were composed of three ASVs belonging to genera *Flavobacterium*, *Parcubacteria* and *Lactobacillus* (Figure 4A and Supplementary Figure 2A). When a less restrictive prevalence threshold was applied (i.e., 80%), 7 ASVs belonging to the genera *Flavobacterium* (2), *Parcubacteria* (1), *Lactobacillus* (2), *Cetobacterium* (1) and *Propionibacterium* (1) could be identified as belonging to the common core (Figure 4B and Supplementary Figure 2B).

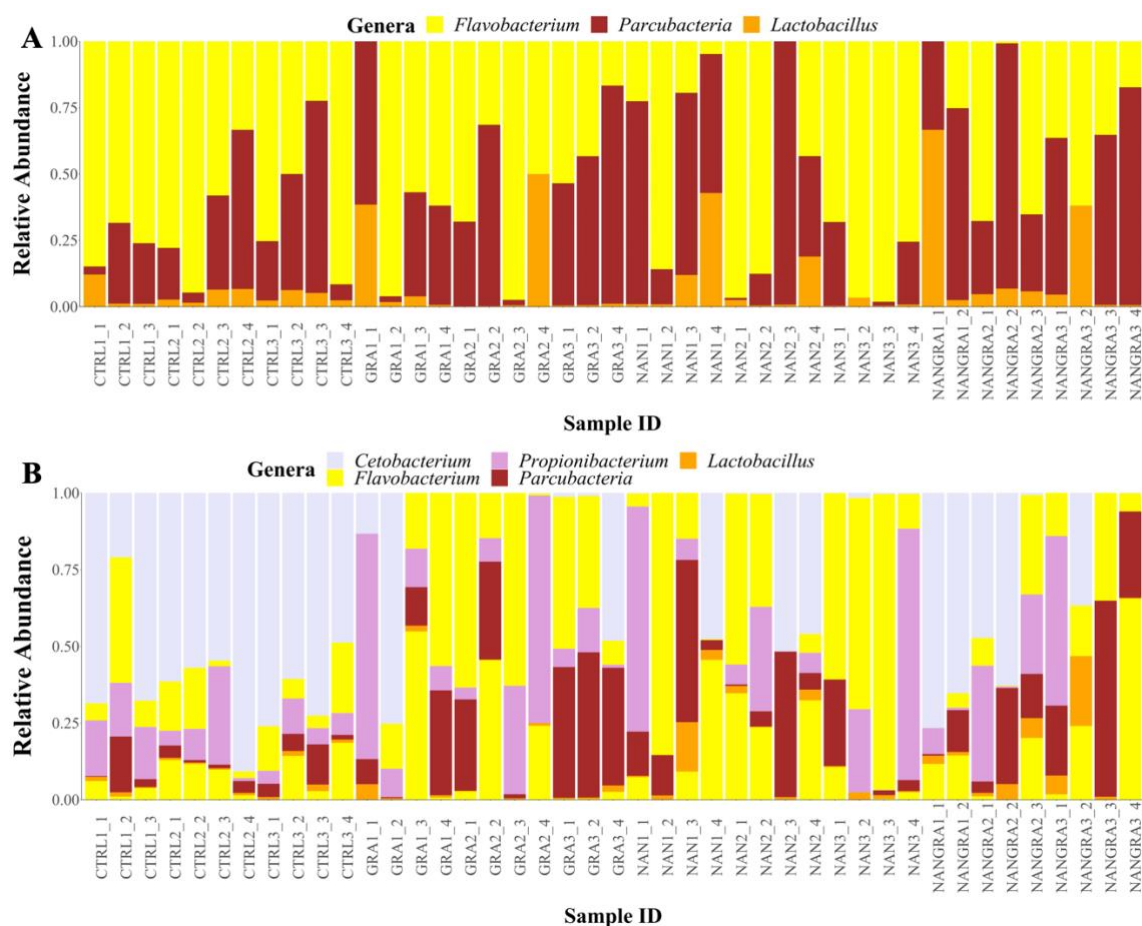


Figure 4. Relative abundance of the core bacterial taxa in the posterior intestine of European seabass fed the experimental diets. (A) Prevalence threshold of 90%; (B) prevalence threshold of 80%. Sample ID: CTRL, control group; GRA, *Gracilaria gracilis* group; NAN, *Nannochloropsis oceanica* group; NANGRA, *G. gracilis* and *N. oceanica* blend group.

3.3.4 Modulation of the bacterial communities by the algae diets

The inclusion of macro- and microalgae, included either singly or in combination, in diets for European seabass led to significant alterations on the abundance of several groups of intestinal bacteria compared to the CTRL-fed fish, as evaluated by DESeq2 (Figure 5). The results indicate that the GRA diet, compared to CTRL, led to an increase (10 to 30-fold changes) in the abundance of *Acinetobacter*, *Corynebacterium*, *Ilumatobacter*, *Kordia*, *Methylobacterium*, *Polaribacter*, *Pseudomonas* and *Sulfitobacter*; and a decrease (10 to 30-fold changes) in the abundance of *Bacillus*, *Exiguobacterium*, *Gp4*, *Gp6*, *Nitrospira*, *Opitutus*, *Rhizobium*, *Roseateles* and *Vampirovibrio* (Figure 5A). Concerning the NAN diet, there was an increase in abundance of ASVs of *Acinetobacter*, *Bacillus*, *Kordia*, *Pseudomonas*, *Rothia*, *Streptococcus* and *Thermicanus* when compared to CTRL (20-fold change); while the abundance of some members of the *Acinetobacter*, *Clostridium sensu strictu*, *Exiguobacterium*, *Guardnerella*, *Muricauda*, *Ruminobacter*, *Vampirovibrio* and

Vibrio was decreased, and the fold changes ranged between -20 and -30 (Figure 5B). The inclusion of the blend (NANGRA diet) led to a proliferation (15 to 25-fold changes) of *Acinetobacter*, *Kordia*, *Massilia*, *Parcubacteria* and *Pseudomonas*, and a decreased representation of ASVs of *Clostridium sensu strictu*, *Gardnerella*, *Muricauda*, *Nitrospira*, *Opitutus*, *Pseudomonas* and *Tepidimonas* (-15 to -25-fold changes) (Figure 5C).

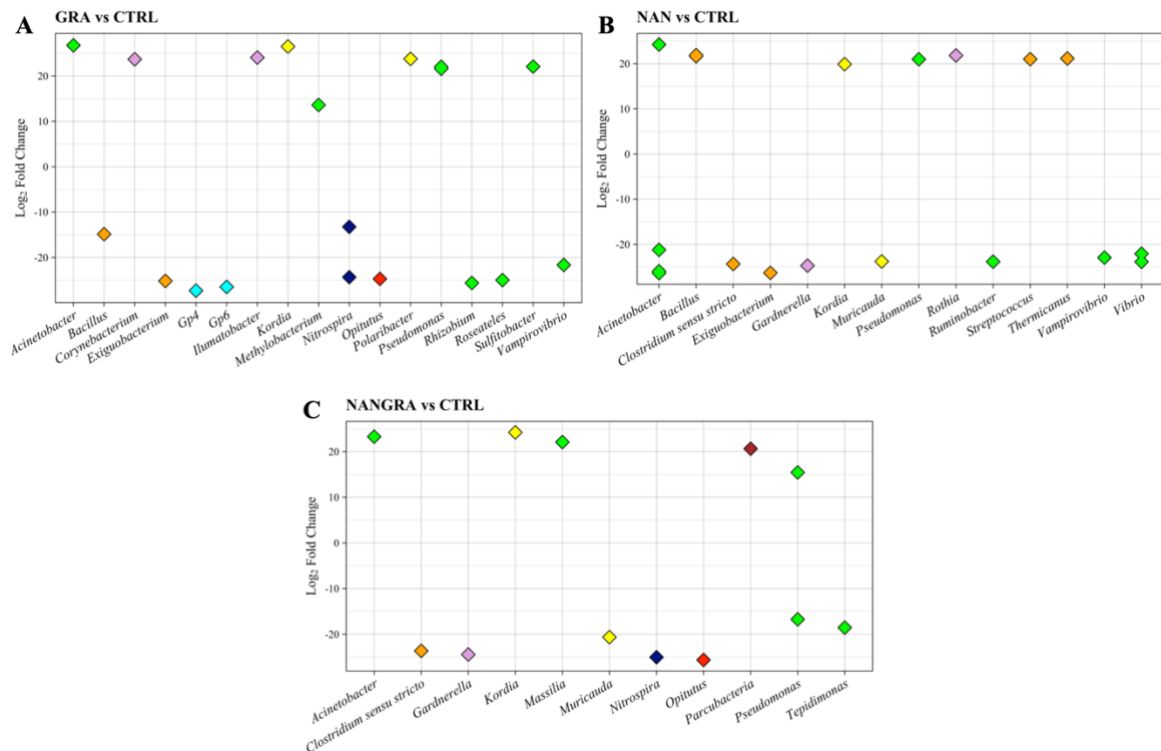


Figure 5. Differently abundant genera in the study groups. (A) GRA group vs CTRL group. (B) NAN group vs CTRL group. (C) NANGRA group vs CTRL group. Color codes: green – Proteobacteria (*Acinetobacter*, *Methylobacterium*, *Pseudomonas*, *Rhizobium*, *Roseateles*, *Sulfitobacter*, *Vampirovibrio*, *Ruminobacter*, *Vibrio*, *Massilia* and *Tepidimonas*); orange – Firmicutes (*Bacillus*, *Exiguobacterium*, *Clostridium sensu strictu*); pink – Actinobacteria (*Corynebacterium*, *Ilumatobacter*, *Gardnerella* and *Rothia*); light blue – Acidobacteria (*Gp4* and *Gp6*); yellow – Bacteroidetes (*Kordia* and *Muricauda*); dark blue – Nitrospirae (*Nitrospira*); light red – Verrucomicrobia (*Opitutus*); dark red – *Parcubacteria*.

3.3.5 Phylogenetic analysis of the ASVs identified as core and those differently modulated by the algae diets

To understand the phylogenetic relationship of certain bacteria which are known to be beneficial and/or pathogenic we first extracted the sequences of the ASVs and performed a blast in NCBI. Thereafter, a phylogenetic tree was constructed to illustrate the genetic relationship, which indirectly indicates functional diversity if there are traits that are retained through evolution.

The phylogenetic tree that includes the ASVs identified as part of the core microbiome in the current study is presented in Figure 6A. The ASVs identified as *Lactobacillus* (ASVs 1679 and 1313) were found to be related to strains of *Lactobacillus delbrueckii* subsp. *lactis*, *Lactobacillus acidophilus*, *Lactobacillus helveticus* and *Lactiplantibacillus plantarum*. The ASV10 *Cetobacterium* clustered with several strains of *Cetobacterium somerae*; while the ASV16 *Propionibacterium* clustered with strains of *Cutibacterium acnes*. The ASVs 11 and 22, identified as *Flavobacterium*, are likely related to a strain of *Flavobacterium succinicans*; whereas ASV19 *Parcubacteria* clustered with the uncultured candidate division OD1 bacterium.

The phylogenetic relationships of another set of ASVs – i.e., those that were significantly modulated by the dietary treatments – with the highly similar sequences from NCBI are presented in Figure 6B. The *Acinetobacter* ASVs 94 and 288 clustered with strains of *Acinetobacter schindleri*; ASV24 appeared in a cluster with strains of *Acinetobacter baumannii*; and ASV43 clustered with strains of *Acinetobacter pittii*. The ASVs identified as *Pseudomonas* clustered together with species of this genus; *Pseudomonas alcaligenes* (ASV35), *Pseudomonas alcaliphila* (ASV37), *Pseudomonas brassicae*, *Pseudomonas putida*, *Pseudomonas japonica* and *Pseudomonas qingdaonensis* (ASV182). *Vibrio* ASV338 clustered with a strain of *Vibrio fortis*; *Ruminobacter* (ASV102) appeared in a cluster with uncultured rumen bacteria; and *Rhizobium* (ASV265) clustered with a strain of *Rhizobium straminoryzae*. *Rothia* (ASV178) is likely related to the strain of *Rothia dentocariosa*; *Streptococcus* (ASV803) clustered with a strain of *Streptococcus parasanguinis*; and the *Bacillus* ASVs clustered with strains of *Geobacillus thermoleovorans* and *Caldibacillus thermoamylovorans* (ASV232) and a strain of *Caldibacillus hisashii* (ASV71). ASV34, annotated as *Kordia*, was phylogenetically close to strains of *Kordia periserrulae* and *Kordia algicida*. *Corynebacterium* (ASV220), *Clostridium sensu stricto* (ASV565), *Exiguobacterium* (ASV240), *Opitutus* (ASV176), *Polaribacter* (ASV113), and *Parcubacteria* (ASV198) clustered with strains of bacteria belonging to same genera.

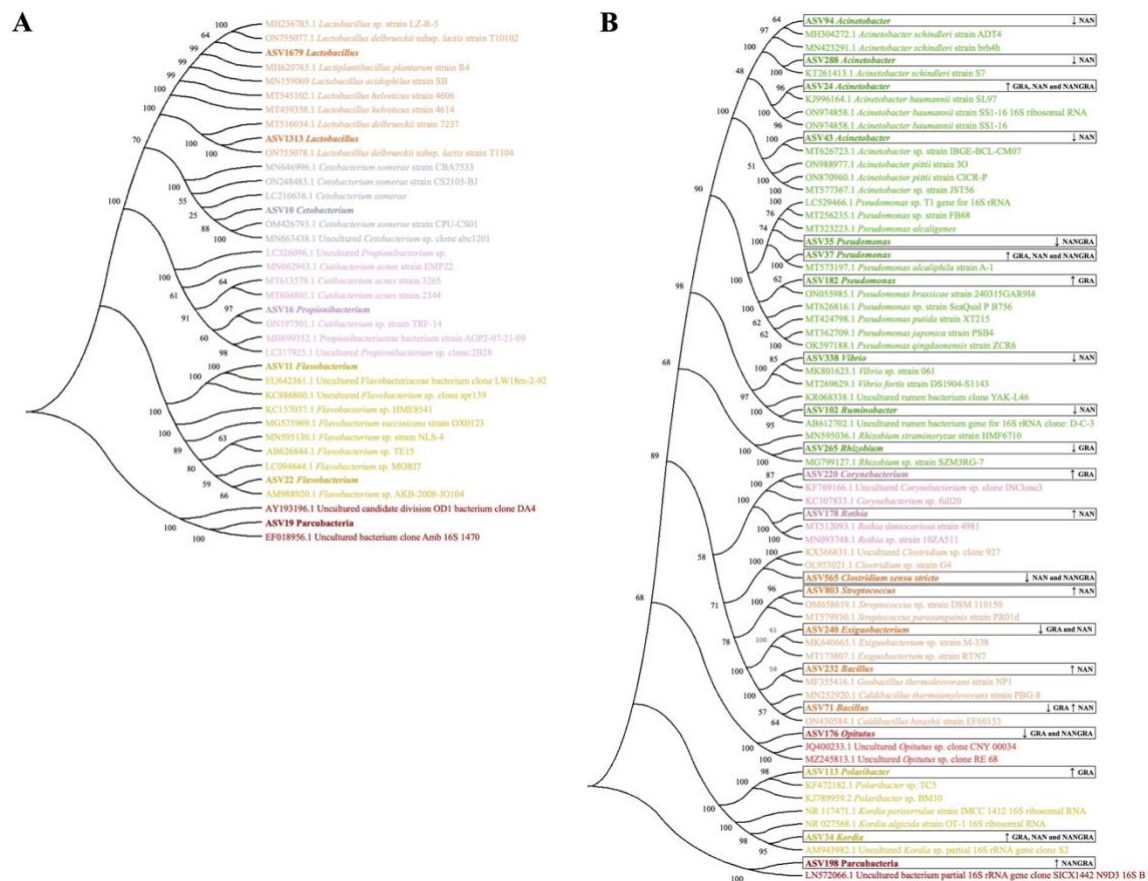


Figure 6. Phylogenetic trees generated for the ASVs of interest identified in the study. (A) ASVs identified as part of the core microbiome. (B) ASVs identified as differently abundant in the study groups. ↑ – increase in abundance; ↓ – decrease in abundance; GRA, *Gracilaria gracilis* group; NAN, *Nannochloropsis oceanica* group; NANGRA, *G. gracilis* and *N. oceanica* blend group.

3.4 Discussion

The gut microbiota of farmed fish has recently become one of the most studied topics in aquaculture research due to the recognized importance of the intestinal bacterial communities to fish health and physiology (Egerton et al., 2018). Although nutrients are known to play a crucial role in modulating the intestine microbiota and algae have been extensively studied in recent years as novel feed ingredients and additives for farmed fish (Hua et al., 2019), the knowledge about the impact of macro- and microalgae on the composition of the intestinal bacteria of fish is still limited (Cerezo et al., 2022; Cerezuela et al., 2012a; Jorge et al., 2019; Keating et al., 2021; Rico et al., 2016; Sagaram et al., 2021). In the present study we evaluated the ability of the macroalga *G. gracilis* and the microalga *N. oceanica*, and a mix of these two algae, to modulate the gut bacterial profile of European seabass, a widely farmed fish species in Europe.

3.4.1 Gut bacterial community composition and diversity in European seabass is affected by the algae diets

The dominant bacterial phyla found in the posterior intestine of European seabass were Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, Fusobacteria, Nitrospirae and Parcubacteria. Our findings are in line with previous studies on this fish species that have also reported the dominance of Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes and Fusobacteria in the gut (Liu et al., 2022b; Pérez-Pascual et al., 2020; Serra et al., 2021). The phylum Nitrospirae has a relatively high representation in our samples from the CTRL group (10.21%), and although it has been previously reported as part of the intestinal microbiota of farmed fish, it was not found among the most abundant phyla in other fish microbiome studies (Li et al., 2019; Serra et al., 2021). As sulfate reducing bacteria, Nitrospirae are usually associated with the water and biofilters of RAS (Fossmark et al., 2021; Minich et al., 2020; Schmidt et al., 2016). Therefore, the presence in the intestine of cultured fish is most likely the result of a transfer event, as also reported by Minich et al. (2020) in Atlantic salmon reared in RAS systems. We did not observe a clear dominance of certain genera across all experimental samples, but *Acinetobacter*, *Nitrospira*, *Pseudoalteromonas* and *Streptococcus* were among the most abundant genera. The genera *Acinetobacter* and *Streptococcus* spp. have strains with probiotic effects (Bunnøy et al., 2019; Swain et al., 2009), but some species are opportunistic bacterial pathogens that cause diseases in hosts that encounter environmental stressors or in immunocompromised fish (Dawood, 2021). On the other hand, *Pseudoalteromonas* species can provide protection against harmful bacteria present in the gut microbiota, namely *Vibrio* (Richards et al., 2017; Rimoldi et al., 2020a). The relatively high abundance of both potentially pathogenic and beneficial bacteria found in the fish intestine denotes the importance of the commensal microbiota in the maintenance of host health, through a tight regulation of competing microorganisms.

The bacterial alpha diversity analysis revealed that the 8% inclusion of *G. gracilis* or *N. oceanica* reduced the species richness (significant only for *G. gracilis*), evenness of the microbial populations and the effective number of dominant species, compared to the CTRL group. Similarly, Faith's phylogenetic diversity, that is a measure of biodiversity based on phylogeny, was also significantly lower in single algae groups. The gut microbial community of European seabass that consumed the seaweed supplemented diet (GRA) also differed significantly from the bacterial community found in the intestine of CTRL-fed fish. Although a decreased bacterial diversity may not always imply an unstable community, a reduction in the microbial diversity and changes in the bacterial profiles in the intestine have been associated with unhealthy fish (Li et al., 2016). Interestingly, inclusion of the algae blend

eliminated these negative effects on the intestinal bacterial diversity, most probably due to the lower inclusion (4%) of each alga. In a complementary study (Batista et al., 2020a), we evaluated the effects of *G. gracilis* and *N. oceanica* on growth performance, nutrient digestibility and intestinal histomorphology; a lower protein digestibility was observed for fish fed the macroalga supplemented diet, but without differences in terms of growth. At the intestine level, the fish on this diet had the lowest number of neutral goblet cells, associated with the digestive and absorptive processes. Similar to the ability bestowed by the microalga in the *G. gracilis*-*N. oceanica* blend to regain the diversity and structure of the intestinal microbiota, our previous study also indicated the positive association of the microalga with the digestion process (Batista et al., 2020a). Together, these results may indicate that inclusion of different micro- and macroalgae in a blend is likely a good strategy to lessen the negative impacts of added-value natural compounds from macroalgae species.

3.4.2 Core microbiome of European seabass

The core microbiota (autochthonous microbiome) is composed of resident microorganisms that colonize the intestinal mucosa (Egerton et al., 2018). In the present study, three and five genera were identified as part of the core microbiota at 90 and 80% prevalence thresholds, respectively. The core bacteria were *Flavobacterium*, *Lactobacillus*, *Parcubacteria*, *Cetobacterium* and *Propionibacterium*. Although part of normal fish microbiota, the genus *Flavobacterium* is well recognized for their opportunistic nature in fish (Derome et al., 2016). Some *Flavobacterium* spp. are responsible for systemic infections that are difficult to control and prevent, and hence, are associated with devastating economic losses that affect the worldwide fish production (Loch & Faisal, 2015). In the present study, the *Flavobacterium* ASVs identified as part of the core microbiome showed a high similarity with a potentially pathogenic bacteria, *F. succinicans*, the likely agent of bacterial gill disease in rainbow trout cultivated in RAS (Good et al., 2015). Interestingly, the genus *Lactobacillus*, belonging to the lactic acid bacteria group, was also identified as part of the core microbiota of European seabass in our study. *Lactobacillus*, well recognized as part of the beneficial gut bacteria, provides protective effects against bacterial infections (Dawood, 2021; He et al., 2017). The core ASVs belonging to *Lactobacillus* clustered with *L. delbrueckii* subsp. *lactis*, *L. acidophilus*, *L. helveticus*, and *Lactiplantibacillus plantarum*, that were reported to have *in vitro* and *in vivo* probiotic activity (Ahire et al., 2019; Hosseini et al., 2016; Iorizzo et al., 2022; Rurangwa et al., 2009).

ASVs assigned to *Parcubacteria* were detected among both core microbiome and the top ASVs in our samples (between 1.3 and 5.6% average relative abundance). Genes encoding for amylases and capacities for degrading cellulose and mannose were associated with

Parcubacteria isolated from sporadic permafrost zone of subarctic Quebec, and this bacteria has the capacity to produce acetate as the major end product of its metabolism (Vigneron et al., 2019). Nonetheless, the role of *Parcubacteria* as part as the gut microbiota of European seabass remains unclear and further studies should delve into understanding the functionality of this bacteria.

As part of the commensal microbiota, *Cetobacterium* and *Propionibacterium* are also often described as beneficial bacteria that are commonly found in healthy fish. Here, the ASVs identified as core and belonging to *Cetobacterium* and *Propionibacterium* were closely related to the strains of *C. somerae* and *Cutibacterium acnes*, respectively. *Cetobacterium* is reported as protease and vitamin B₁₂ producing bacteria (Legrand et al., 2020), and previous studies have demonstrated the ability of the fermentation products of *C. somerae* to improve gut health of different farmed fishes (Xie et al., 2022; Xie et al., 2021b; Zhou et al., 2022). The genus *Propionibacterium* - producer of propionate, linolenic acid, vitamins, and antimicrobials - is known to enhance host robustness by boosting the immune response and promoting the growth of other probiotic bacteria (Boutin et al., 2013; Zárate, 2012). In gilthead seabream *Propionibacterium* has also been reported as an important genus of the core intestinal microbiota, producing metabolites with an important role for gut health (Piazzon et al., 2017; Piazzon et al., 2019).

The presence of taxa, namely *Lactobacillus* and *Cetobacterium*, with probiotic potential among the highly prevalent taxa in majority of the studied population indicate that these bacteria play a key role in the maintenance of biological functions, health, and disease resistance in European seabass.

3.4.3 Algae, singly or in combination, modulate both beneficial and potentially pathogenic groups

The intestinal microbiota of farmed fish is a complex assemblage of microorganisms that interact and compete for nutrient acquisition and space. Both beneficial and potentially pathogenic groups of bacteria coexist in the gut, with nutrition playing a leading role in the modulation of these bacterial communities (Dawood, 2021; Tarnecki et al., 2017). In the present study, macro- and microalgae feeds affected the abundance of several groups of bacteria present in the posterior intestine of the seabass when compared to fish fed the CTRL diet.

The inclusion of *G. gracilis* and *N. oceanica*, fed both singly and blended, led to a significant increase in the representation of ASVs belonging to potentially pathogenic bacteria. Notably there was an increased representation of an ASV that clustered with strains of *Acinetobacter baumannii*; a strain of this bacterium was previously recovered from diseased

channel catfish (*Ictalurus punctatus*) (Xia et al., 2008). The GRA diet led to an increase in a *Pseudomonas* ASV that clustered together with *P. putida*, a bacteria known to cause disease in both humans and fish (Mao et al., 2013). *Rothia*, that proliferated in the NAN group, has only been reported as an opportunistic pathogen in humans (Fatahi-Bafghi, 2021). As fish may act as a vehicle for the transmission of drug-resistant human pathogens, it is of crucial importance to understand the impact of novel formulations on the fish microbial communities. Other groups of potentially pathogenic bacteria that were significantly increased only in the gut of GRA-fed fish included *Corynebacterium* (Tarnecki et al., 2018) and *Polaribacter*, a potential RAS-associated pathogen (Rud et al., 2017). It is noteworthy that some *Polaribacter* species are associated with important antioxidant functions (Sehna et al., 2021). A pro-oxidative or bacterial challenge could give further insights into the impact of these potentially pathogenic genera on fish health.

The *Gracilaria* diets (GRA and NANGRA) were associated with a significant decrease of the genus *Nitrospira*. During the 15-weeks feeding trial, all abiotic conditions, including the nitrogenous compounds in the RAS system, were carefully monitored, and optimum levels of water quality parameters were maintained in the rearing system of the fish. Therefore, the lower proportion of the genus *Nitrospira* (the phylum Nitrospirae) found in macroalgae groups is most likely a response to the dietary treatment. Suo et al. (2017) studied the impact of sulfide exposure on gut microbiota of Pacific white shrimp (*Litopenaeus vannamei*) and reported that although present in the intestine of non-exposed shrimp, Nitrospirae could not be found in sulfide-exposed groups. The authors argued that the disappearance of this bacterial phylum upon sulfide exposure could make shrimp more susceptible to nitrite toxicity. *Nitrospira* spp. are nitrite-oxidizing bacteria than convert nitrite into the less toxic nitrate (Philips et al., 2002). Therefore, the reduction of *Nitrospira* in intestine of European seabass fed *G. gracilis* diets might negatively impact the fish ability to cope with a nitrite exposure situation. Nitrite uptake in marine fish is thought to occur through the gills and intestine, and although it is less toxic for marine fish, previous studies have found negative impacts of elevated nitrite levels on growth (Ciji & Akhtar, 2019).

The algae-rich diets modulated certain groups of bacteria involved in nutrient degradation and utilization. The genus *Opitut*, that are cellulose degraders commonly found on surfaces of green periphytic algae (Knack et al., 2015), was less abundant in seaweed-rich diets. Likewise, a decreased abundance of bacteria belonging to the genus *Rhizobium* that are known to have cellulolytic and pectolytic activity (Xia et al., 2018), was observed in the GRA group. Macroalgae, much like plants, have cellulose-based walls (Kumar et al., 2013). Therefore, these bacterial groups may aid in the degradation of the algae and plant products present in the experimental diets, and a decrease in its abundance may partially explain the lower nutrient digestibility associated with the *Gracilaria* diets, as reported by Batista et al.

(2020a). Indeed, the authors attributed the reduced protein and energy digestibility in fish fed the GRA and NANGRA diets to the presence of indigestible fibers in the macroalga cell wall that compromised the action of the digestive enzymes. The involvement of the gut microbiota in the digestive/absorptive processes in fish has been previously recognized (Butt & Volkoff, 2019), and the above-mentioned results further support these interactions. Although the genera *Ruminobacter*, described as starch utilizer (Darabighane et al., 2021), and *Clostridium sensu stricto*, previously characterized as a carbohydrate degrader (Abdelhamed et al., 2019), had a lower abundance in the intestine of fish fed the *Nannochloropsis* diet, in our associated study (Batista et al., 2020a) we did not find a difference in nutrient or energy digestibility in the NAN group compared to the CTRL group. Moreover, fish fed the algae supplemented diets had an increased representation of an ASV that was annotated as *Kordia*, that is likely closely related to *K. periserrulae* and *K. algicida*. Members of *K. algicida* can exhibit algicidal activity and produce extracellular proteases responsible for the cell lysis of diatoms in a species-specific way (Demuez et al., 2015). Therefore, a higher abundance of *Kordia* in the intestine of European seabass may aid in the digestion of microalgae enriched diets. On the other hand, the genus *Exiguobacterium* was found to be less abundant in not only the NAN-fed fish but also the GRA-fed fish. A previous study on zebrafish has found that *Exiguobacterium* sp. play a role in lipid droplet formation in enterocytes to positively impact the fatty acid absorption (Semova et al., 2012). Further studies on the function of this bacterial genus on fish gut microbiota are required, but the reduced abundance of *Exiguobacterium* in single alga diets may indicate an impact of these algae on the metabolism of dietary fat. Nonetheless, there were no differences between the lipid digestibility and whole-body and muscle fat contents of fish that consumed algae-rich diets and those fed the CTRL diet (Batista et al., 2020a). A modulation of both beneficial (i.e., increase) and potentially pathogenic (i.e., decrease) bacteria was observed in the intestine of European seabass fed the macro- or microalgae diets. Inclusion of seaweed *G. gracilis* in feeds led to a higher abundance of *Sulfitobacter* and *Methylobacterium* (producer of poly- β -hydroxybutyrate that degrades short-chain fatty acids), two genera that comprise bacteria capable of inhibiting the growth of fish pathogens (Boutin et al., 2013; Wilczynski et al., 2022). Similarly, the *N. oceanica* feeds led to an increase in beneficial bacteria such as *Bacillus* in fish intestine. Spore-forming species such as *Bacillus* spp. are probiotics with wide application in the aquaculture industry. The *Bacillus* ASVs that were enriched in the gut of NAN-fed fish clustered together in the phylogenetic tree with bacterial strains such as *Geobacillus thermoleovorans*, *Caldibacillus thermoamylovorans* and *C. hisashii* that exhibit antimicrobial and probiotic activities (Inabu et al., 2022; Zebrowska et al., 2022). Spore-forming bacterial species belonging to the genus *Bacillus* have been previously isolated from the intestine of European seabass, with

some isolates presenting carbohydrase activity and probiotic potential (Serra et al., 2019). From an industrial perspective, it would be interesting to further explore the ability of the microalga *N. oceanica* to enrich the gut of European seabass with *Bacillus* spp. with promising probiotic activity. Although limited information on the topic is available, some studies have reported that species belonging to the genus *Acinetobacter*, namely *Acinetobacter lwoffii*, *Acinetobacter junii* and *A. pittii* are emerging fish pathogens and their virulence against fish of the genus *Schizothorax* was also revealed previously (Cao et al., 2018; Malick et al., 2020). Interestingly, the NAN diet led to a decrease in several *Acinetobacter* ASVs, including an ASV that likely has a close phylogenetic relationship with the pathogen *A. pittii*. Although the genus *Pseudomonas* is often reported as part of the normal microbiota and certain bacteria belonging to this taxa are probiotic strains (Qi et al., 2020), there are also opportunistic fish pathogens among them that are responsible for disease outbreaks and high mortality in farms (Oh et al., 2019). NANGRA-fed fish presented a reduced abundance of an ASV belonging to *Pseudomonas* that is probably related to *P. alcaligenes*, a rare but potentially opportunistic fish pathogen (Bai et al., 2021). This microalga also decreased the abundance of the genus *Vibrio*, one of the most important fish pathogens responsible for devastating economic losses in fish farms. In the present study, the ASV of the genus *Vibrio* clustered with a strain of *Vibrio fortis*, a bacterium with reported pathogenicity against rainbow trout (Austin et al., 2005), and that has been found in association with enteritis in cultured seahorses (*Hippocampus erectus*) (Wang et al., 2016). *In vitro* studies have recently demonstrated the antimicrobial activity of *G. gracilis* and *N. oceanica* against some *Vibrio* species, namely *V. harveyi* and *V. parahaemolyticus* (Ferreira et al., 2021). Disease outbreaks caused by *Vibrio* are bottlenecks for the long-term sustainability of the aquaculture sector. In this context, microbiota modulation using added-value products such as those employed in this study may be a promising strategy to improve disease resistance in fishes.

3.5 Conclusion

In the present study it was demonstrated for the first time the potential of the seaweed *G. gracilis* and the microalga *N. oceanica*, incorporated singly or blended in European seabass diets, to modulate the intestinal microbiota of the fish. It was observed that 8% inclusion of the two algae (singly) led to a reduction of the gut microbial diversity, which is often associated with a negative impact. Nonetheless, inclusion of a lower percentage of each alga in a blend (4% each) was able to mitigate these plausible effects on the intestinal bacterial communities. The core microbiome of European seabass was composed of both beneficial (*Lactobacillus* and *Cetobacterium*) and potentially pathogenic (*Flavobacterium*)

bacteria, which might suggest that the host gut homeostasis and disease resistance is dependent on a tight interaction between competing microorganisms. The algae-rich feeds modulated some groups of bacteria that are known carbohydrate degraders, with the GRA fish showing a decreased abundance of the genera *Opitutus* and *Rhizobium*, which may partially explain the lower nutrient digestibility observed in fish that consumed the macroalgae diet. *Gracilaria* promoted the growth of bacteria capable of outcompeting fish pathogens (*Sulfitobacter* and *Methylobacterium*); while *Nannochloropsis* led to a higher representation of *Bacillus* bacteria, widely recognized for their probiotic potential, and a decreased abundance of the potentially pathogenic bacteria belonging to the genus *Acinetobacter*. NAN-fed fish also presented a lower abundance of ASVs identified as *Vibrio*, a highly pathogenic bacteria affecting the culture of several farmed aquatic animals of high economic value. Such results are indicative of the potential of algae to modulate the bacterial communities present in the intestine of European seabass, with possible implications to host health, nutrition, and disease resistance.

3.6 Data availability statement

The data used in this study is available at Sequence Read Archive (SRA) with accession number PRJNA867546.

3.7 Author contributions

LMPV and VK were responsible for funding acquisition and the conceptualization of the study. MF and YA performed the formal analysis and investigation. HA and JS provided the resources for the study. MF wrote the original draft under the supervision of LMPV and VK, who reviewed and edited it. All authors have read, revised, and agreed on the final version of the manuscript.

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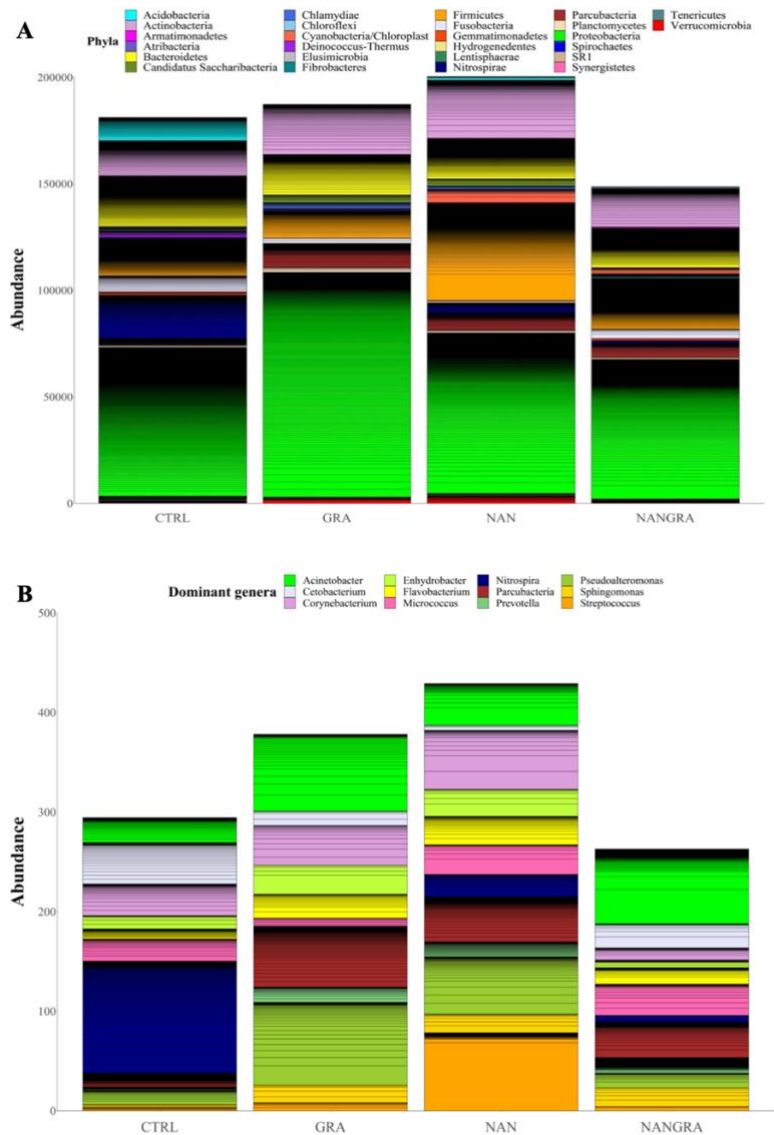
3.9 Acknowledgements

The support received from Bisa Saraswathy, Researcher, Nord University, for data analyses and preparation of the manuscript is acknowledged.

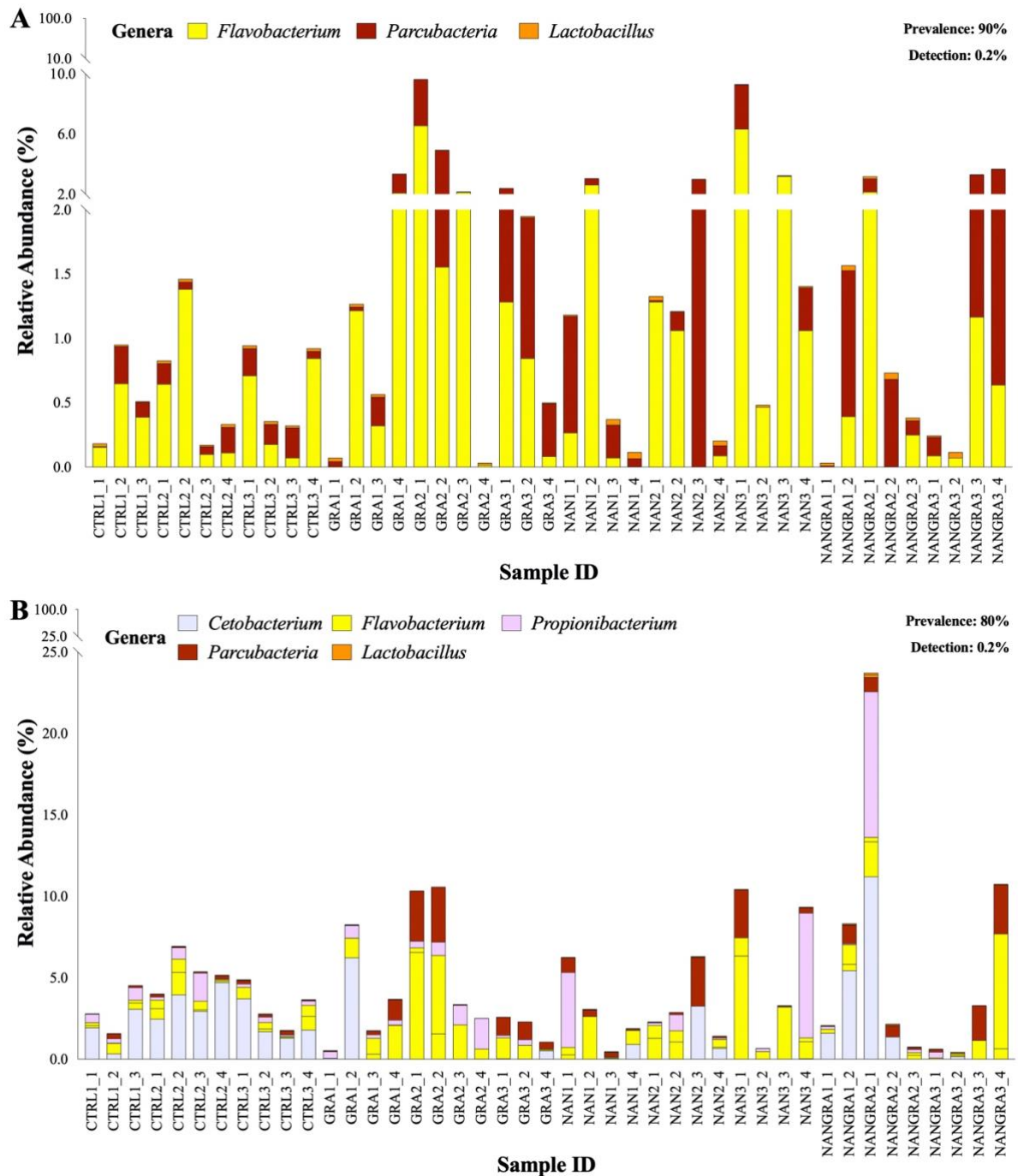
3.10 Conflict of interest

HA and JS were employed by the companies ALGApplus and Allmicroalgae, respectively. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

3.11 Supplementary material



Supplementary Figure 1. Abundances of the bacterial phyla (A) and dominant genera (B), per dietary treatment, in the posterior intestine of European seabass.



Supplementary Figure 2. Relative abundance of the core bacterial taxa in the posterior intestine of European seabass fed the experimental diets. (A) Prevalence threshold of 90%; (B) prevalence threshold of 80%.

Chapter 4.

Inclusion of algae blend in European seabass diets: Impacts on intestinal histomorphometry and microbiota

By

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Abstract

The need to produce high-quality seafood and the potential of functional feeds to maintain fish health underscore the importance of natural value-added products like algae. Within the aquaculture community, there is a growing focus on maintaining intestinal health through dietary interventions, as preserving intestinal functions is key to fish performance. We evaluated the impact of an algae blend-supplemented feed on the intestinal histomorphometry and microbial communities of European seabass. Our study employed a control group (CTRL) and three blend diets (Blends 2, 4, and 6) with 2%, 4%, and 6% algae inclusion, respectively. Blend-fed fish had increased muscularis thickness, villi length, submucosa width and a higher number of acid goblet cells compared to CTRL fish, suggesting that this algae blend can positively affect intestinal motility, nutrient absorption, and mucosal immunity. The intestinal bacterial community of European seabass was dominated by Proteobacteria, while the second-most prevalent phyla varied in the dietary groups. *Delftia* emerged as the most prevalent genus, indicating the intricate dynamics of the seabass gut microbiota. Although microbial diversity remained relatively stable across groups, the intestine of fish fed the Blend 2 diet exhibited significant alterations in specific bacterial genera compared to the other experimental diets, including *Burkholderia*, *Streptococcus*, *Acinetobacter*, *Enterobacter*, *Corynebacterium*, *Brevundimonas*, *Rhizobium*, *Tepidimonas*, *Anoxybacillus* and *Plesiomonas*. In summary, our study demonstrates the significant impact of the algae blend on seabass intestinal structure and microbial composition. However, effects depend on inclusion levels, and specific bacterial alterations may not align with changes in intestinal structure, growth, and immune responses.

Keywords: seaweeds, microalgae, European seabass, gut histology, intestinal microbiota

4.1 Introduction

Functional feeds have become key inputs in modern aquaculture because they offer a versatile approach to improve both fish health and performance (Olmos Soto et al., 2015). These specialized feeds are intended to provide beneficial effects beyond those imparted by essential nutrients when they are formulated with bioactive compounds, probiotics, prebiotics (Dawood et al., 2018), and even unconventional ingredients like micro- and macroalgae (Valente et al., 2021; Wan et al., 2019). As our understanding of the intricate relationship between functional feeds and fish health continues to evolve, these feeds hold promise in optimizing aquaculture practices, ensuring the production of healthier and more resilient farmed fish, and addressing the growing global demand for sustainable seafood. Algae, in this context, emerge as promising functional ingredients for aquafeeds due to their well-documented bioactive properties (Eroldoğan et al., 2023). While much of the research in aquaculture nutrition has focused on the use of algae as feed ingredients (Li et al., 2022; Rico et al., 2016; Valente et al., 2006), there is also a growing body of knowledge regarding their role as feed additives for various farmed fish species (Cerezo et al., 2022; Ferreira et al., 2023b; Peixoto et al., 2019). However, most attention has been devoted to the incorporation of single micro- and macroalgae, for example microalgae like *Nannochloropsis oceanica* and *Chlorella vulgaris*, as well as macroalgae like *Gracilaria gracilis* and *Ulva rigida*, which are already produced at an industrial scale (Ferreira et al., 2021). Collectively, the available literature emphasizes the potential of algae to enhance fish growth performance, nutrient digestibility (Mota et al., 2023), and disease and stress resistance (Ferreira et al., 2023b; Magnoni et al., 2017; Peixoto et al., 2019). In line with current trends in aquaculture research, both academia and industry are increasingly recognizing intestinal health as a critical element that positively contributes to overall fish performance and indirectly upholds sustainability of the industry. Consequently, dietary interventions aimed at preserving both intestinal morphology and the composition of microbial communities, which are linked to intestinal health, have become a prominent target of marketed functional feeds (Dawood, 2021; Hoseinifar et al., 2019). By modulating key morphological features of the intestine (e.g., villi length, goblet cells number, etc.) and promoting the growth of beneficial bacteria, these functional feeds aid in nutrient absorption, disease resistance, and overall well-being of the fish (Dawood, 2021; Ferreira et al., 2023c). Indeed, when reviewing the existing literature, it is clear that algae can have notable effects on fish gut histomorphology and microbial communities. Studies have reported a decrease in intestinal microbial diversity when different fish species were fed microalgae, seaweeds, or algae-derived compounds (Cerezuela et al., 2012a; Ferreira et al., 2022; Gupta et al., 2019). Such dietary interventions also have the potential to increase the abundance of

beneficial bacteria while reducing the potentially pathogenic bacteria, indicating the effectiveness of algae to ultimately influence nutrient utilization, immune responses, and disease resistance (Ferreira et al., 2022; Gonçalves et al., 2022; Gupta et al., 2019). Additionally, the incorporation of algae in feeds can result in alterations in key morphological features of the intestine, such as villi length, goblet cell numbers, and the overall gut structure (Batista et al., 2020a; Cerezuela et al., 2012a; Mota et al., 2023; Sørensen et al., 2021). The research focus on exploring the impact of algae blends in aquafeeds by leveraging their diverse nutritional and bioactive properties is aimed at creating value-added feeds (Batista et al., 2020a; Ferreira et al., 2023c; Mota et al., 2023). Nonetheless, more comprehensive knowledge about the effects of algae blend as functional ingredients for fish is imperative for the formulation of more effective and health-promoting aquafeeds.

This is particularly true for species such as European seabass (*Dicentrarchus labrax*), which is an economically relevant food fish in the Mediterranean region and is frequently faced with production challenges due to disease outbreaks. Thus, studies evaluating the functionality of novel feeds for this fish species are now more relevant than ever. In our previous studies, we discovered that a blend of micro- and macroalgae (*Nannochloropsis oceanica* and *Gracilaria gracilis*) could modulate the bacteria present in the posterior intestine of European seabass without affecting the intestinal microbial diversity (Ferreira et al., 2022). We have also shown that a commercially available algae blend (ALGAESSENCE™ – Feed), consisting of microalgae *N. oceanica* and *C. vulgaris* and macroalgae *G. gracilis* and *U. rigida*, can enhance growth (Mota et al., 2023), immune responses, and disease resistance in this species (Ferreira et al., 2023b). Hence, in the present study, we aim to investigate whether the benefits of this commercial algae blend are related to changes in the gut structure and microbial communities in the posterior intestine of European seabass.

4.2 Materials and methods

4.2.1 Ethical statement

The animal experiments were carried out by certified scientists authorized to conduct laboratory research with animals, adhering to the guidelines set forth by the European Union (Directive 2010/63/EU). The protocols received approval from the animal welfare committee of CIIMAR (Centro Interdisciplinar de Investigação Marinha e Ambiental; ORBEA-CIIMAR 06-2016) and they were also overseen by the Portuguese Veterinary Authority.

4.2.2 Experimental diets, feeding trial, and sampling

The complete description of the experiment design, feeding trial, and zootechnical data can be found in Mota et al. (2023). The experimental diets included a commercial algae blend (ALGAESSENCE™ – Feed) produced at an industrial scale in Portugal by Allmicroalgae and ALGAplus. The algae blend is composed of two microalgae (*Nannochloropsis oceanica* and *Chlorella vulgaris*) and two macroalgae (*Gracilaria gracilis* and *Ulva rigida*). Four diets were formulated, all with the same levels of protein (53% dry matter) and energy (22 kJ/g DM). The control diet (CTRL) had high levels of vegetable protein sources (71.8%) and moderate fish meal content (12.5%). The three experimental diets (Blend 2, 4, and 6) had 2%, 4%, and 6%, of the commercial algae blend, respectively, replacing whole peas and wheat gluten. Further information on the chemical composition of the algae blend and the experimental diets can be found in Ferreira et al. (2021) and Mota et al. (2023) respectively. The feeding trial was conducted at the CIIMAR animal facility in Matosinhos, Portugal. Briefly, European seabass juveniles (average weight of 11.3 ± 2.7 g) were distributed into twelve 50-liter fiberglass tanks of a saltwater recirculation system. Throughout the trial, water conditions in the tanks of the study groups remained consistent, with water temperature at 20.9 °C, salinity of 35‰, pH of 7.5 and a photoperiod regime of 12-hour light/12-hour dark. The experimental diets were randomly assigned to each tank, with three tanks per diet. Fish were fed thrice daily until they appeared satiated, and the feeding trial lasted for 12 weeks (84 days).

At the end of the feeding trial, and following a 24-hour fasting period, fish were humanely euthanized with a swift blow to the head. The gastrointestinal tract of six fish from each tank (18 fish per treatment) was excised under sterile conditions. Specifically, the posterior intestine, which is the section immediately before the ileorectal valve as described in Ferreira et al. (2023c), was carefully opened with sterile scissors. Mucus from this section was collected using a sterile swab (Copan Italia, Italy). The swab was then placed into a sterile tube and promptly frozen in liquid nitrogen. These samples were stored at -80 °C until further analysis. Additionally, a small 1 cm segment from the posterior region of the intestine was collected, thoroughly cleaned, and fixed in a 4% formaldehyde solution with a pH of 7.0 ± 0.1 for subsequent histological assessment.

4.2.3 Histomorphological evaluation of the posterior intestine

Posterior intestine from four fish per tank were selected (12 fish per dietary treatment) for the histological analysis. After the tissue fixation, the samples were preserved in 70% ethanol. They were subsequently subjected to standard histological procedures and embedded in paraffin. Intestinal cross-sections measuring 3 µm were obtained with a semi-

automated rotary microtome (Leica RM 2245) and they were then stained with Alcian Blue/Periodic Acid Schiff (AB/PAS; pH 2.5). Micrographs of each section were examined using a light microscope (Olympus BX51, Germany) equipped with a camera (Olympus DP50).

The analysis of the sections, each representing one cross-section per intestinal sample, was conducted using a quantitative approach as outlined in (Ferreira et al., 2023c). Specifically, one section per sample was examined using imaging software (Olympus cellSens Standard 2.2) to assess various parameters, including the cross-sectional perimeter, the absorption area, villi length, muscularis thickness, submucosa thickness, lamina propria width, and the number of goblet cells, which encompassed both acid and neutral GC. The total count of acid and neutral GC, as well as the absorption area (representing the total area occupied by the villi), was automatically determined using the analysis tools provided by the Olympus cellSens software, in accordance with the procedures outlined in Ferreira et al. (2023c).

4.2.4 DNA extraction for 16S rRNA sequencing

All the procedures detailed here were carried out under sterile conditions. For DNA extraction, six samples from each treatment were randomly selected and distributed into four separate batches. Genomic DNA was isolated from these samples employing the QIAamp DNA stool Mini Kit (Qiagen, Germany), following the manufacturer's protocol with some adjustments, but following the procedures described in Ferreira et al. (2022). Briefly, swabs with mucus from the posterior intestine were placed in tubes containing beads and InhibitEX buffer (Qiagen). Afterward, samples were homogenized using the Precellys Evolution tissue homogenizer, followed by centrifugation. The resulting pellet was resuspended and incubated at 70 °C for 20 min. After incubation, samples were mixed and centrifuged, and the resulting supernatant was mixed with proteinase K. The remaining steps of the DNA extraction followed the manufacturer's protocol for the QIAamp DNA stool Mini Kit. The DNA was eluted using 75 µL of ATE buffer, and its quantity was assessed using the Qubit dsDNA Assay (Thermo Fisher Scientific Inc., USA).

4.2.5 Amplicon library preparation and sequencing

The amplicon library preparation and sequencing followed the protocols described in Ferreira et al. (2022). Briefly, the initial PCR reaction focused on the hypervariable V3-V4 region of the 16S rRNA gene, using primers 341F (5' CCTACGGGNGGCWGCAG 3') and 805R (5' GACTACNVGGGTWTCTAATCC 3') (Klindworth et al., 2013), which were flanked by Illumina adapters (Illumina, USA). The PCR reactions were performed in triplicate using

10 μ M of each primer, 5 ng/ μ L of DNA template and Amplitaq Gold Q5 (Thermo Fisher Scientific Inc.). Negative PCR controls, devoid of DNA template, were also included in the experiment. Following amplification, the replicates of each sample were combined for subsequent procedures. The amplified products were purified using the CleanNGS system (CleanNA, Netherlands), following the manufacturer's instructions. The index PCR was performed using the purified products and Nextera XT Index primers (Illumina), by adopting the conditions described by the manufacturer. After purification, the quality of the obtained libraries was assessed using a Tapestation 2200 platform (Agilent Technologies, USA), and the libraries were subsequently quantified using the Quant-IT PicoGreen dsDNA assay kit (Thermo Fisher Scientific Inc.). After pooling, the library was quantified by Real-Time qPCR, employing the KAPA Library quantification kit (Roche). The libraries were then sequenced on an Illumina MiSeq (PE300) platform, which utilized MiSeq Control Software 2.5.0.5 and Real-Time Analysis software 1.18.54.0.

4.2.6 Sequence data analysis

Raw sequence data has been submitted to the Sequence Read Archive (SRA) under the accession number PRJNA1031113. As previously described in Ferreira et al. (2022), the initial step involved truncating the obtained reads to a length of 270 base pairs using VSEARCH (Rognes et al., 2016). Next, MICCA pipeline (v1.7.2) (Albanese et al., 2015) was employed for further data processing. Within MICCA, the criteria for merging the sequences were a minimum overlap length of 60 base pairs and a maximum allowable mismatch of 20 base pairs. Any reads lacking primers were excluded, and both forward and reverse primers were removed from the merged reads. Following this, reads with an expected error rate exceeding 0.75 (Edgar & Flyvbjerg, 2015) were filtered out, and sequences shorter than 400 base pairs were discarded.

The MICCA pipeline utilized the "de novo UNOISE" method - UNOISE3 algorithm by Edgar (2016) - to perform denoising on the filtered reads. This process involves the correction of sequencing errors and the determination of true biological sequences at single-nucleotide precision. Subsequently, any chimera and mitochondrial sequences were eliminated, and the resulting dataset was used to generate amplicon sequence variants (ASVs) for subsequent analyses. Taxonomies were assigned to the bacterial ASVs using the RDP classifier. Sequence alignment was accomplished through the NAST multiple sequence aligner (DeSantis et al., 2006), and a phylogenetic tree was constructed using the FastTree software integrated within the MICCA pipeline.

4.2.7 Statistical analysis

Statistical analysis of the histological data was conducted using the IBM SPSS software platform version 27.0 (SPSS Inc., Chicago, IL, USA). The normality and homogeneity of variances were assessed through Kolmogorov–Smirnov and Levene's tests, respectively, with transformations applied when needed. The dietary treatments were compared using a one-way ANOVA, followed by a Tukey's multiple comparison test ($P < 0.05$).

The statistical analysis regarding the microbiota data were conducted within R studio version 1.4.1103. The "iNEXT" and "phyloseq" packages were utilized to compute alpha diversity metrics, which included overall species richness (utilizing the "ChaoRichness" function), Shannon diversity (employing the "ChaoShannon" function), and Simpson diversity (with the "ChaoSimpson" function). The "pd" function from the "picante" package was employed to assess Faith's phylogenetic diversity. The Kruskal-Wallis test, followed by Dunn's test, was applied to evaluate dissimilarities between the experimental groups. Subsequently, the functions in "ggplot2," was employed to create the graphical representations for overall species richness, Shannon diversity, Simpson diversity, and Faith's phylogenetic diversity (Hsieh et al., 2016).

Bacterial beta diversity analysis utilized the weighted UniFrac distances (Lozupone & Knight, 2005), and beta diversity was visualized through a principal coordinates analysis (PCoA) plot. The dispersion of microbial communities was initially assessed using the "betadisper" function, and subsequently, significant differences between the communities were determined through the application of Permutational Multivariate Analysis of Variance Using Distance Matrices (PERMANOVA) involving 9,999 permutations (Anderson, 2001). This analysis was carried out by employing the "adonis" function within the vegan R-package (Oksanen et al., 2013), followed by pairwise comparisons. The identification of differentially abundant ASVs involved an analysis of the non-rarefied data (McMurdie & Holmes, 2014) using the "DESeq2" package, the function of which enables the normalization of the data (Love et al., 2014).

4.2.8 Phylogenetic analysis

Phylogenetic analysis of the ASVs identified as differentially abundant was performed as described by Ferreira et al. (2022). Briefly, Blastn was firstly used to compare the identified ASV sequences with bacterial sequences in the NCBI Reference Sequence Database, selecting sequences that had a 100% match. The matched sequences were then imported into Molecular Evolutionary Genetics Analysis (MEGAX) software for multiple sequence alignment using the ClustalW aligner. Phylogenetic analysis employed the unweighted average binding among clusters (UPGMA) method with default parameters and 500

bootstrap replicates. Finally, the resulting phylogenetic trees were visualized using MEGAX software.

4.3 Results

4.3.1 Histomorphology of the intestine of European seabass fed the algae blend diets

To assess the impact of the algae blend on posterior intestine histomorphology, we conducted a quantitative analysis, and the results are presented in Table 1. Fish that were fed the blend diets exhibited significantly higher final body weights compared to the CTRL group. This improved growth performance coincided with several alterations in gut structure. Specifically, there was a significant increase in the cross-sectional perimeter of the posterior intestine in the Blend 4 and Blend 6 groups compared to the CTRL group. Similarly, the muscularis thickness, total number of goblet cells, and the number of acid goblet cells were all elevated in fish that consumed the 4% and 6% algae blend diets compared to the CTRL group. The submucosa width showed an increase in all blend groups, while villi length was higher only in the Blend 6 group compared to the CTRL group.

Table 1. Histomorphology of the posterior intestine of European seabass fed either the CTRL or the algae blend-supplemented diets (Blend 2, 4 and 6).

	CTRL	Blend 2	Blend 4	Blend 6	P-value
Final body weight (g)*	37.3 ± 9.9 ^c	54.4 ± 15.4 ^b	56.6 ± 16.4 ^b	62.9 ± 16.1 ^a	< 0.001
Cross-sectional perimeter (mm)	7.2 ± 1.0 ^b	8.1 ± 0.8 ^{ab}	8.9 ± 1.2 ^a	8.5 ± 0.7 ^a	0.004
Absorption area (mm ²)	1.7 ± 0.4	2.0 ± 0.4	2.1 ± 0.6	2.0 ± 0.2	0.09
Muscularis thickness (μm)	77.8 ± 21.3 ^c	100.5 ± 19.5 ^{bc}	128.1 ± 21.3 ^a	109.9 ± 17.5 ^{ab}	< 0.001
Submucosa width (μm)	28.9 ± 7.2 ^b	42.6 ± 6.3 ^a	43.0 ± 10.2 ^a	49.3 ± 5.3 ^a	< 0.001
Lamina propria width (μm)	14.1 ± 4.4	14.1 ± 3.0	15.5 ± 4.8	13.5 ± 3.0	0.7
Villi length (μm)	506.93 ± 53.3 ^b	571.1 ± 85.3 ^{ab}	583.1 ± 66.4 ^{ab}	600.7 ± 59.5 ^a	0.03
Goblet cells (GC)	1780.9 ± 354.1 ^b	2069.9 ± 681.3 ^{ab}	2310.9 ± 448.5 ^a	2548.2 ± 372.9 ^a	0.03
Acid GC	1595.9 ± 280.1 ^b	1947.4 ± 708.2 ^{ab}	2202.4 ± 496.9 ^a	2389.6 ± 484.1 ^a	0.03
Neutral GC	185.0 ± 105.3	122.4 ± 72.3	108.4 ± 162.6	164.7 ± 162.4	0.6

Values presented as mean ± SD (n = 12 samples per dietary treatment). Different letters denote significant differences between dietary treatments. *Data retrieved from Mota et al. (2023).

4.3.2 Microbial composition of the intestine of European seabass fed the algae blend diets

All the samples were analysed together in one MiSeq run, producing a total of 44,940,528 high-quality reads, with an average of 191,562 reads per sample. To account for variations in read counts among the samples, the data were rarefied to 45,000 per sample. Out of the initial 72 samples, four were removed due to low read counts. Thus, 68 samples remained for subsequent analysis: 18 from the CTRL group and 17 for each of the algae blend groups. After processing, a total of 1,496 ASVs were identified across all samples, spanning 19 phyla and 205 genera.

Regarding the populations of intestinal bacteria in European seabass, the 19 bacterial phyla identified are illustrated in Figure 1A, showing their relative abundance per sample. The most abundant phyla, displayed in Figure 1B, included Actinobacteria, Bacteroidetes, Cyanobacteria/Chloroplast, Firmicutes, Fusobacteria, Proteobacteria and Tenericutes. Across all the different dietary groups, there was a clear prevalence of the phylum Proteobacteria, with an average relative abundance of 72.3% in the CTRL group, 70.6% in the Blend 2 group, 84.0% in the Blend 4 group, and 75.0% in the Blend 6 group. In the CTRL, Blend 2 and Blend 6 groups, the second most abundant phylum was Tenericutes (14.5, 21.0, and 5.4%, respectively). On the other hand, in Blend 4 group, Firmicutes appeared as the second most prevalent phylum, with an average relative abundance of 4.4%. The third most prevalent phyla were Actinobacteria for all blend groups (3.3, 3.8 and 5.2% for Blend 2, 4 and 6, respectively) and Firmicutes for the CTRL group (3.8%) (Table 2).

The dominant genera specific to each dietary treatment are presented in Figure 1C and Table 2. These dominant genera included *Acinetobacter*, *Anoxybacillus*, *Aquabacterium*, *Bacillus*, *Cetobacterium*, *Corynebacterium*, *Delftia*, *Neisseria*, *Propionibacterium*, *Staphylococcus*, *Stenotrophomonas* and *Streptococcus*. The proportions of the dominant genera varied according to the dietary treatment, but a clear dominance of the genus *Delftia* was observed across the intestinal samples (Figure 1C). Indeed, *Delftia* appeared as the most prevalent genus, with an average relative abundance of 61.8% in the CTRL group, 61.6% in the Blend 2 group, 71.7% in the Blend 4 group, and 58.7% in the Blend 6 group. The second most prevalent genus was, for all dietary groups, *Stenotrophomonas*, with an average relative abundance that ranged from 2.6 to 3.7%. The genus *Cetobacterium* appeared as the third most prevalent genus in the Blend 4 and Blend 6 groups (3.4 and 2.6%), but for the CTRL and Blend 2 groups, the third most prevalent genera were *Streptococcus* (1.8%) and *Corynebacterium* (2.1%), respectively.

Table 2. Average relative abundance (%) of the dominant bacterial phyla and genera present in the posterior intestine of European seabass fed either the CTRL or the algae blend-supplemented diets (Blend 2, 4 and 6).

	CTRL	Blend 2	Blend 4	Blend 6
Phyla				
Actinobacteria	2.3 ± 0.8	3.3 ± 9.8	3.8 ± 2.6	5.2 ± 5.9
Bacteroidetes	2.2 ± 0.7	1.4 ± 3.4	1.3 ± 3.4	2.4 ± 3.3
Cyanobacteria/Chloroplast	0.4 ± 0.8	0.08 ± 0.1	0.3 ± 0.9	1.8 ± 5.4
Firmicutes	3.8 ± 1.2	1.5 ± 3.3	4.4 ± 9.2	0.2 ± 0.5
Fusobacteria	1.5 ± 1.5	1.0 ± 2.8	3.4 ± 7.6	2.6 ± 3.6
Proteobacteria	72.3 ± 12.2	70.6 ± 32.6	84.0 ± 22.5	75.0 ± 24.9
Tenericutes	14.5 ± 7.3	21.0 ± 31.8	3.1 ± 8.7	5.4 ± 10.6
Genera				
<i>Acinetobacter</i>	0.5 ± 1.3	0.1 ± 0.2	0.3 ± 0.4	2.5 ± 5.8
<i>Anoxybacillus</i>	0.02 ± 0.1	0.02 ± 0.1	1.9 ± 5.0	0.1 ± 0.4
<i>Aquabacterium</i>	0.3 ± 0.3	0.4 ± 0.4	0.3 ± 0.3	0.5 ± 0.7
<i>Bacillus</i>	0.2 ± 0.5	0.3 ± 0.7	0.4 ± 1.0	0.9 ± 2.5
<i>Cetobacterium</i>	1.4 ± 2.7	0.9 ± 2.7	3.4 ± 7.6	2.6 ± 3.6
<i>Corynebacterium</i>	0.5 ± 1.1	2.1 ± 8.4	0.7 ± 1.5	0.9 ± 1.5
<i>Delftia</i>	61.8 ± 33.6	61.6 ± 34.6	71.7 ± 30.4	58.7 ± 33.7
<i>Neisseria</i>	0.9 ± 2.2	0.0002 ± 0.0008	0.5 ± 1.9	1.6 ± 2.6
<i>Propionibacterium</i>	0.6 ± 1.8	0.01 ± 0.02	0.3 ± 0.6	1.0 ± 1.6
<i>Staphylococcus</i>	0.9 ± 2.9	0.6 ± 2.1	0.3 ± 0.6	1.0 ± 1.7
<i>Stenotrophomonas</i>	2.6 ± 2.1	2.8 ± 1.7	3.7 ± 1.8	2.8 ± 1.9
<i>Streptococcus</i>	1.8 ± 3.9	0.005 ± 0.02	0.2 ± 0.4	1.2 ± 2.1

Values presented as mean ± SD.

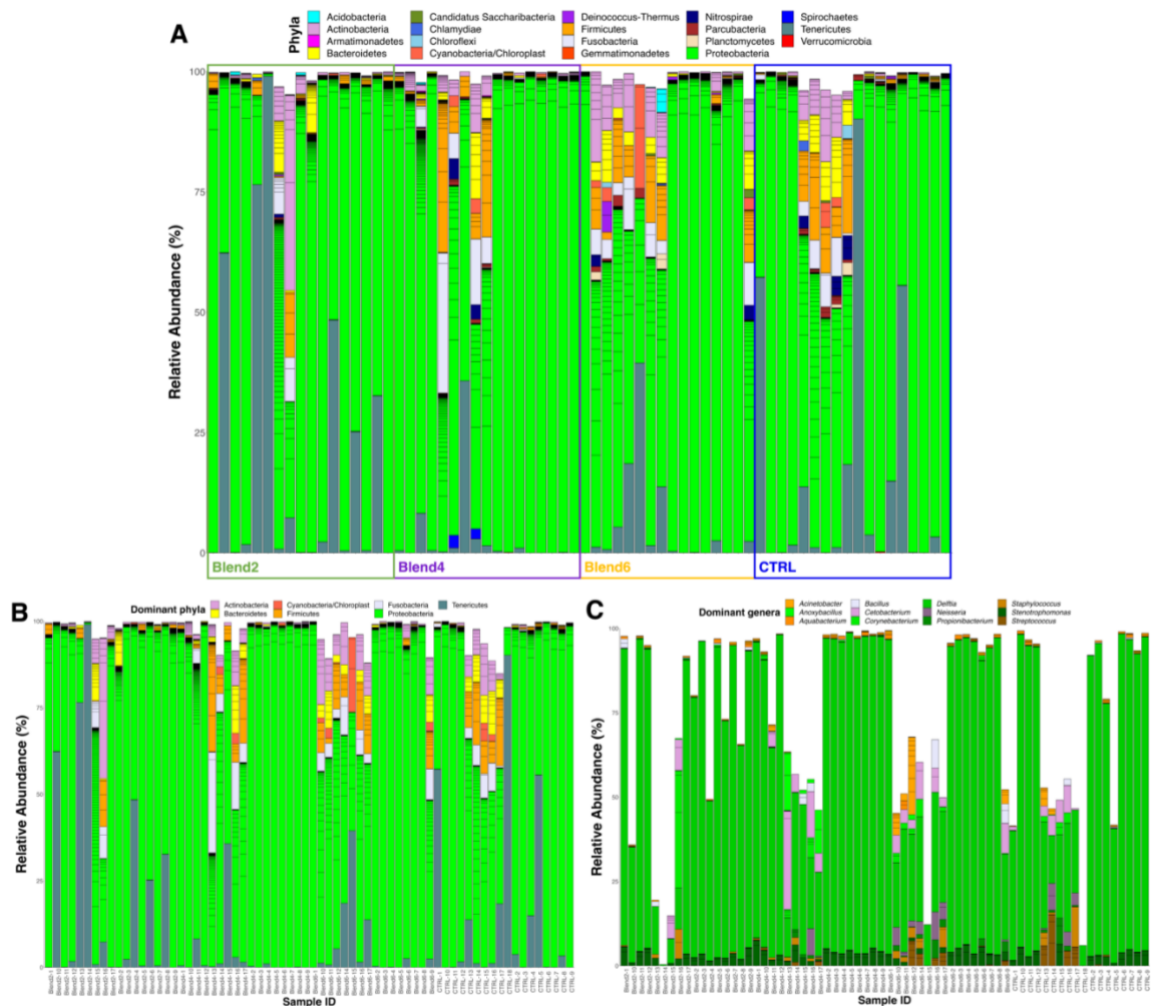


Figure 1. Relative abundance of the bacterial phyla (A), dominant phyla (B) and dominant genera (C) within the posterior intestine of European seabass fed either the CTRL or the algae blend-supplemented diets (Blend 2, 4 and 6). Unclassified bacteria are not included in the figures.

4.3.3 Intestinal microbiota diversity analysis

An analysis of alpha diversity, which is depicted in Figure 2A, was performed using ecological diversity metrics such as Chao richness (a measure of species richness), Shannon diversity (indicating the even distribution of microbes), and Simpson diversity (indicating the number of dominant species). No significant differences among the dietary treatments were found regarding species richness (Figure 2A1), Shannon diversity (Figure 2A2), or Simpson diversity (Figure 2A3). Similarly, Faith's phylogenetic diversity showed similarities across all groups (Figure 2B).

For beta diversity analysis, we employed the weighted UniFrac distance metric to understand the differences in community structure. The principal coordinates analysis (PCoA) plot showed that the first two components explained 23.3% and 11.1% of the variance in the dataset. The results from the PERMANOVA test indicated that there were

no significant differences in the intestinal bacterial communities of the CTRL and Blend groups (Figure 2C).

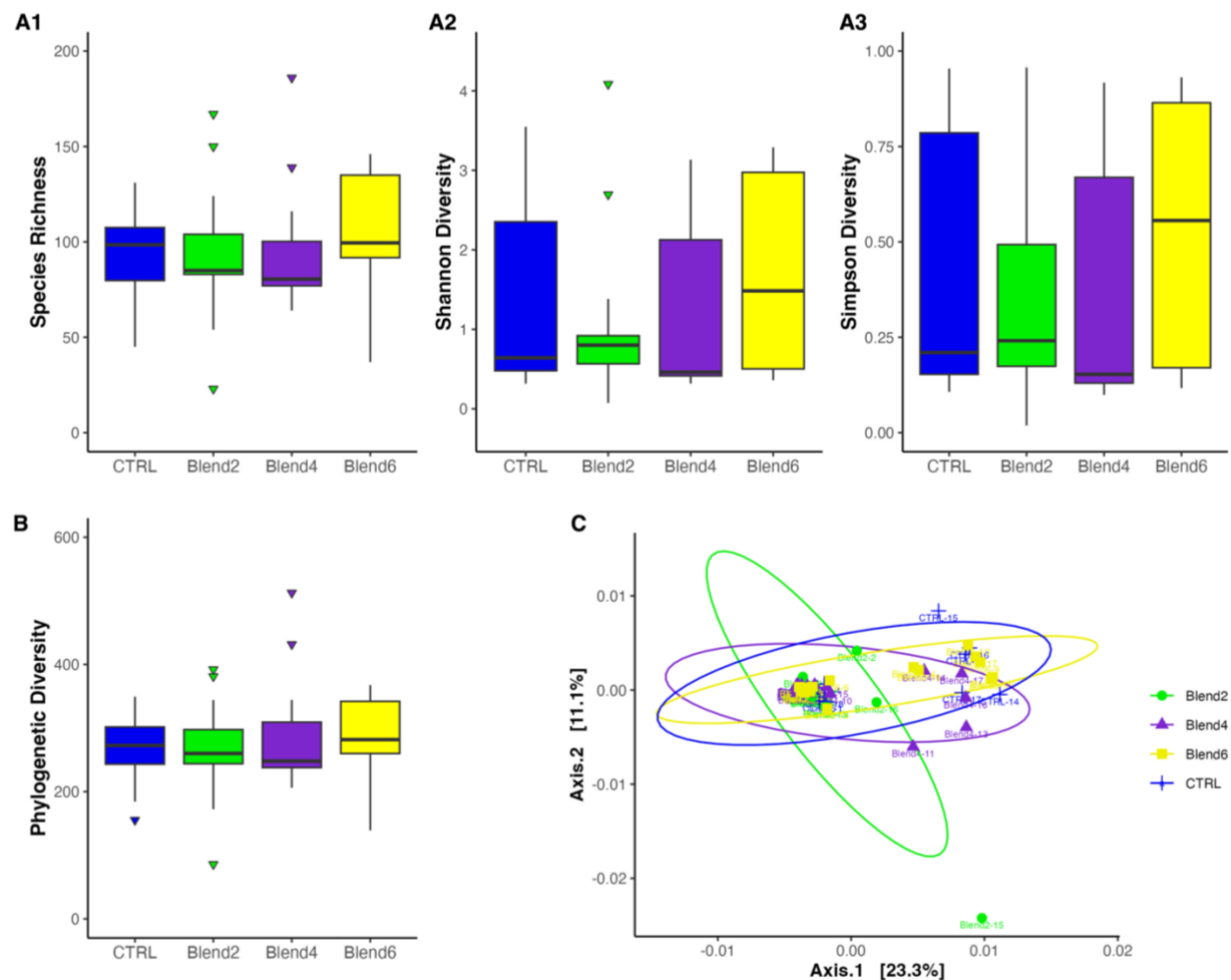


Figure 2. Diversity of the bacterial communities within the posterior intestine of European seabass fed either the CTRL or the algae blend-supplemented diets (Blend 2, 4 and 6). (A) Measures of alpha diversity are displayed, including species richness (A1), Shannon diversity (A2), and Simpson diversity (A3). (B) Faith's phylogenetic diversity. (C) Beta diversity visualized through a principal coordinate analysis (PCoA) plot using weighted UniFrac distances.

4.3.4 Modulation of the bacterial communities by the algae blend diets

The analysis yielded significant changes in the abundance of specific groups of bacteria present in the intestine of European seabass. These changes were evident when comparing the data of the CTRL, Blend 4, and Blend 6 groups to those of the Blend 2 group. Consequently, comparisons were solely conducted between Blend 2 and the other experimental groups (Figure 3). Further analysis of ASVs' sequences from these distinct

bacteria was conducted to establish their phylogenetic relationship, offering insights into their potential functional diversity (Figure 4).

The Blend 4 group vs Blend 2 group comparison revealed that the substantial increase (ranging from 13 to 23-fold change) in the abundance of bacteria such as *Acinetobacter*, *Burkholderia*, *Corynebacterium*, *Granulicatella*, *Haliscomenobacter*, *Neisseria*, *Streptococcus*, *Streptophyta*, *Tepidimonas* and *Vogesella*. On the other hand, there was a decrease (ranging from -16 to -20-fold change) in the abundance of *Enterobacter*, *Runella* and *Sulfurimonas* (Figure 3A). The Blend 6 diet increased the representation of several groups of bacteria compared to blend 2 diet, specifically *Burkholderia*, *Corynebacterium*, *Enterobacter*, *Haliscomenobacter*, *Neisseria*, *Runella*, *Streptococcus*, *Streptophyta*, *Sulfurimonas* and *Vogesella*, with a 13 to 25-fold change (Figure 3B). Compared to the Blend 2 group, fish that were fed the CTRL diet had a higher abundance of certain bacterial groups, namely *Acinetobacter*, *Brevundimonas*, *Burkholderia*, *Enterobacter*, *Granulicatella*, *Haliscomenobacter*, *Neisseria*, *Rhizobium*, *Streptococcus*, *Sulfurimonas* and *Vogesella*, with fold changes ranging from 14 to 26. On the other hand, there was a lower representation of specific bacterial groups in the CTRL group compared to the Blend 2 group, including *Anoxybacillus*, *Plesiomonas* and *Streptophyta*, with fold changes ranging from -17 to -20 (Figure 3C).

The phylogenetic tree displays the ASVs with varying abundance among the study groups (Figure 4). Some ASVs, specifically ASV185 (*Acinetobacter*) related to *Acinetobacter radioresistens*, ASV96 (*Tepidimonas*) clustering with strains of *Tepidimonas arfidensis*, and ASVs 47 and 352 (*Corynebacterium*) associated with non-identified species within this genus (Figure 4), were uniquely modulated by the Blend 4 diet. The ASV12 (*Corynebacterium*), exclusively influenced by Blend 6, is likely associated with strains of *Corynebacterium sanguinis* and *Corynebacterium lipophiloflavum*. The ASVs exclusively modulated by CTRL consisted of the following: ASV190, which appeared in a cluster with *Acinetobacter baumannii*; ASV230, related to strains of *Brevundimonas vesicularis*; ASV42, clustering with strains of *Rhizobium leguminosarum*; ASV21, clustering with uncultured *Streptococcus* sp.; ASV262, associated with *Plesiomonas shigelloides* strains; and ASV62 (*Anoxybacillus*), appearing in a cluster with unidentified *Bacillus* sp.

Several ASVs responded to two or more of the tested diets. ASV115 (*Granulicatella*) increased in both Blend 4 and CTRL and was associated with *Granulicatella adiacens*. ASVs 93 and 52 (found in higher abundance in Blend 6 and CTRL) clustered with strains of *Streptococcus salivarius* and *Streptococcus sanguinis*, respectively. ASV58 (*Runella*) increased in Blend 6 but decreased in Blend 4, aligning with uncultured and unidentified species. Some ASVs exhibited higher representation across all groups (CTRL, Blend 4, and Blend 6) compared to the Blend 2 group. These included ASV20, clustering with *Vogesella*

perlucida; ASV106, associated with *Burkholderia puraquae* and *Burkholderia contaminans*; ASV5, linked to *Neisseria perflava*; ASV17, found in a cluster with *Streptococcus pseudopneumoniae* and *Streptococcus mitis* strains; and ASV26, related to an unidentified *Haliscamenobacter* species. ASVs 34 and 38, found in a cluster with *Enterobacter cloacae* and uncultured *Sulfurimonas* species, respectively, increased in Blend 6 and CTRL groups but decreased in Blend 4. Meanwhile, ASV102 (*Streptophyta*), with higher representation in Blend 4 and 6 but lower presence in CTRL, clustered with *Hydrocotyle wilfordii* and *Hydrocotyle sibthorpioides* (Figure 4).

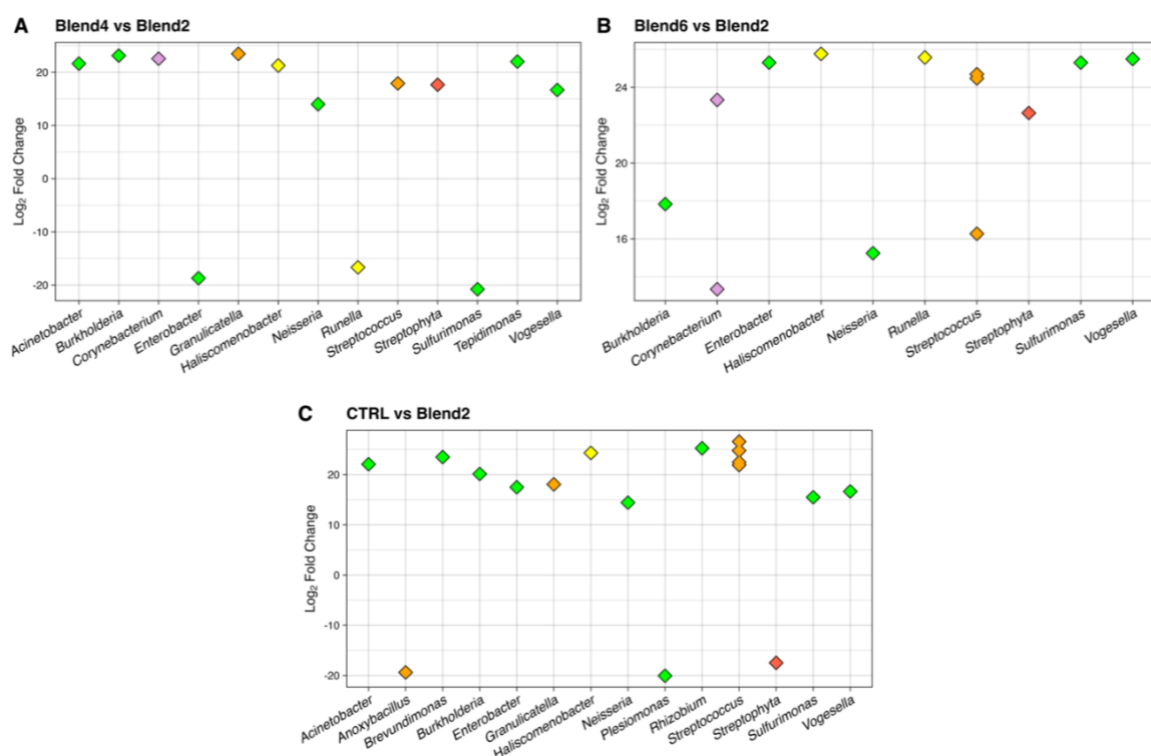


Figure 3. Differently abundant genera in the study groups. (A) Blend 4 group vs Blend 2 group. (B) Blend 6 group vs Blend 2 group. (C) CTRL group vs Blend 2 group. Colour codes: green – Proteobacteria (*Acinetobacter*, *Brevundimonas*, *Burkholderia*, *Enterobacter*, *Neisseria*, *Plesiomonas*, *Rhizobium*, *Sulfurimonas*, *Tepidimonas* and *Vogesella*); orange – Firmicutes (*Anoxybacillus*, *Granulicatella* and *Streptococcus*); pink – Actinobacteria (*Corynebacterium*); yellow – Bacteroidetes (*Haliscamenobacter* and *Runella*); light red – Cyanobacteria/Chloroplast (*Streptophyta*).

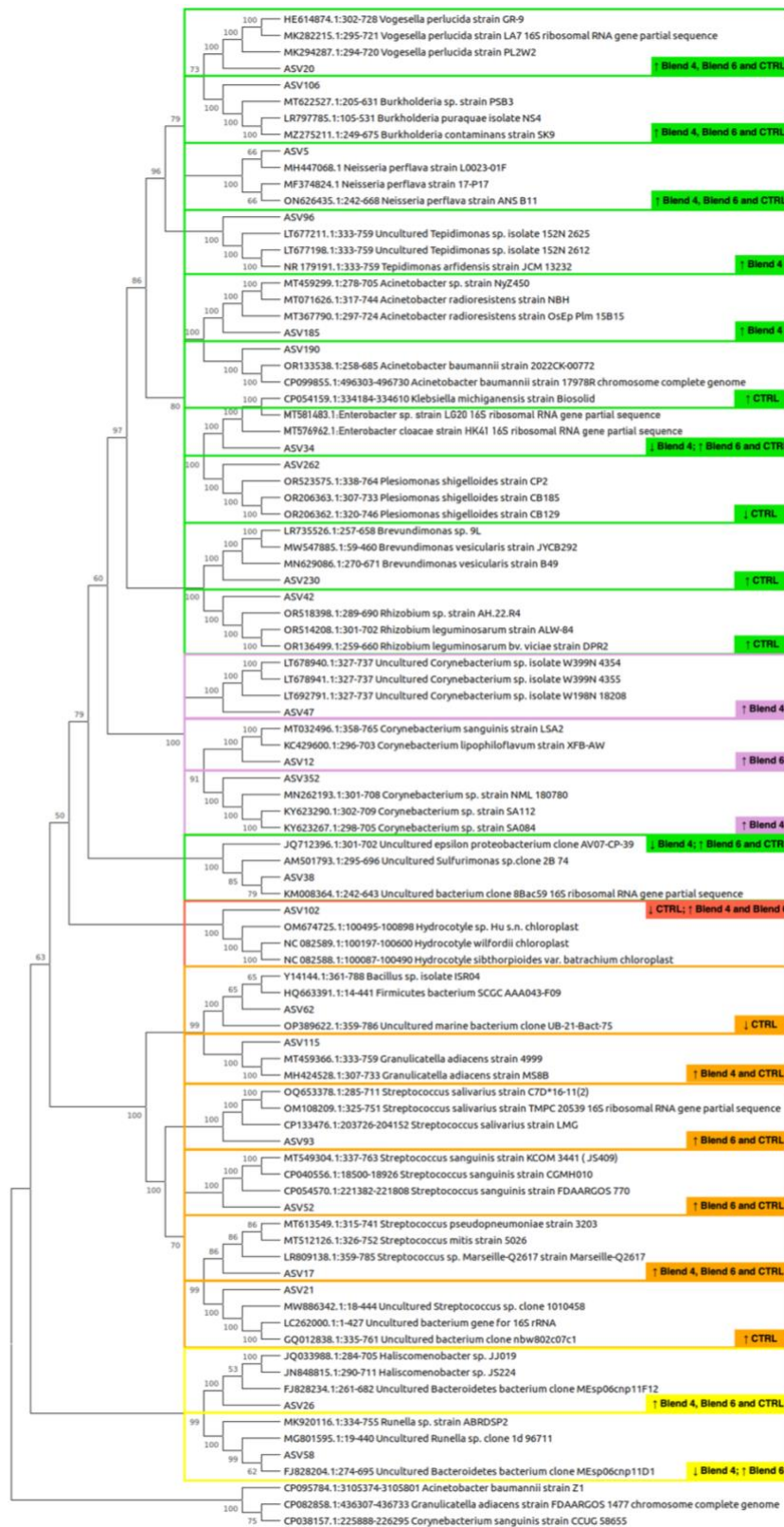


Figure 4. Phylogenetic tree representing the ASVs highlighted as displaying varying abundance among the study groups. Arrows ↑ - increase in abundance; ↓ - decrease in abundance.

4.4 Discussion

The structure and microbiota of the intestine are pivotal in preserving host health and physiology (Tarnecki et al., 2017). This also holds true for European seabass, a commercially significant species in the Mediterranean area. The recognition of the significance of the intestinal bacterial community for the health of farmed fish has propelled research in this field to the next phase; investigating the impact of dietary factors, particularly algae blends on these readouts (Batista et al., 2020a; Ferreira et al., 2022). In the present study, we assessed how an algae blend, consisting of microalgae *N. oceanica* and *C. vulgaris* and macroalgae *G. gracilis* and *U. rigida*, impacts the gut histomorphology and microbiota of European seabass, and provide a specific discussion on the link between these outcomes and their potential effects on fish health and performance.

With the growing popularity of functional feeds in the market, nutritionists have turned their attention to intestinal indices because of their usefulness as valuable indicators of fish health and welfare. Indeed, the significance of the intestine in terms of fish nutrition, immunity, and overall performance is now widely acknowledged (Dawood, 2021; Kuz'mina, 2021). To assess the impact of the algae blend on fish intestinal health, we conducted a quantitative analysis, focusing on specific intestinal histomorphological parameters, which have a bearing on mucosal immunity (Ferreira et al., 2023c). As demonstrated in a previous complementary study, fish fed the algae blend diets exhibited significantly higher final body weights compared to the CTRL diet (Mota et al., 2023). This improved growth performance coincided with several alterations in gut structure. Notably, the increased villi length in Blend 6-fed fish might indicate a higher absorptive capacity of these fish. Although the anterior intestine is commonly associated with nutrient digestion and absorption, certain critical absorption processes, such as water absorption, take place in the posterior segments (Calduch-Giner et al., 2016; Ferreira et al., 2023c; Giffard-Mena et al., 2007). The observed increase in muscularis thickness in fish that consumed the 4% and 6% algae blend diets indicates improved intestinal motility, which is especially important in the distal intestine for efficient waste release (Ferreira et al., 2023c; Olsson & Holmgren, 2001). Additionally, the higher number of acid goblet cells in Blend 4 and 6-fed fish and the increased submucosa width in all blend groups suggest enhanced mucosal immunity promoted by the algae blend. Indeed, previous studies have highlighted the significance of acid goblet cells in protecting against intestinal insults, such as pathogen invasion (Ferreira et al., 2023b; Kim & Khan, 2013). The findings of this study illustrate the capacity of the algae blend, particularly when incorporated into European seabass diets at 4% and 6% inclusion levels, to have a positive impact on growth performance and histomorphological parameters of the posterior intestine.

These results indicate that the blend functions as a functional ingredient with the potential to improve fish health and physiology.

The analysis of gut bacterial composition in the posterior intestine of European seabass revealed a diverse array of bacterial phyla across all dietary treatments, with a clear dominance of Proteobacteria. These results are consistent with our previous study in which seabass were fed with micro- and macroalgae, either singly or in combination (Ferreira et al., 2022). Noteworthy is the dominance of Proteobacteria in both studies, but the relative abundance ranged from 35% to 53% in the previous study compared to 70% in all groups of the present study. The second most prevalent phylum varied among the dietary groups, with Tenericutes, Firmicutes, and Actinobacteria also found to be playing significant roles in European seabass fed the experimental diets. Previous research has indicated that Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes are among the most predominant phyla in the gut of European seabass (Ferreira et al., 2022; Gatesoupe et al., 2016; Gonçalves et al., 2022; Nikouli et al., 2018; Pérez-Pascual et al., 2020; Serra et al., 2021). However, Tenericutes, which emerged as the second most prevalent phylum in all dietary groups except Blend 4, is not detected among the top phyla of the intestinal bacterial community of marine fish. Nevertheless, it has been previously identified as part of the autochthonous microbiota of European seabass (Rimoldi et al., 2020b) and other marine farmed fish species, such as *Solea senegalensis* (Tapia-Paniagua et al., 2019).

At the genus level, we identified a diverse array of dominant genera, with *Delftia* being the most prevalent genus, with a relative abundance ranging from 59% to 72% in the study groups. Notably, bacteria belonging to the genus *Delftia* did not appear among the dominant genera in our previous study (Ferreira et al., 2022). However, it has been previously reported as part of the core microbiota of European seabass (Nikouli et al., 2018). The genera *Stenotrophomonas* and *Cetobacterium* emerged as the second and third most prevalent groups, respectively, with relative abundances ranging from 1.4% to 3.7%. Serra et al. (2021) have also identified the genus *Stenotrophomonas* as part of the core microbiota of European seabass, and it is commonly found in intestinal, skin, and gill samples from this fish species (Gonçalves et al., 2022; Rosado et al., 2019; Torrecillas et al., 2017a). Although the genus *Stenotrophomonas* includes emergent fish pathogens like *Stenotrophomonas maltophilia*, it has also been identified on the skin of apparently healthy individuals (Rosado et al., 2019). Additionally, *Stenotrophomonas* species have been associated with the regulation of nitrogen and sulfur cycles and have been identified as possible agents for bioremediation (Ryan et al., 2009). Regarding *Cetobacterium*, we have previously identified this genus as one of the most prevalent in the posterior intestine of European seabass, and it appeared as part of the core bacterial taxa in previous studies (Ferreira et al., 2022; Rimoldi et al., 2020b). The genus *Cetobacterium* is linked to enhanced gut health in farmed

fish due to the probiotic properties of certain species within this genus. There is also evidence about their ability to produce vitamin B12, antimicrobial metabolites, and volatile fatty acids (Xie et al., 2022; Zhao et al., 2023; Zhou et al., 2022).

The alpha diversity analysis revealed that there were no significant differences among the dietary treatments in terms of species richness, evenness, or the number of dominant species. Similarly, Faith's phylogenetic diversity showed similarities across all dietary groups, and the beta diversity analysis did not indicate any significant differences in the bacterial communities found in the intestine of the CTRL and algae blend groups. It is worth noting our observation in a previous study; the inclusion of *G. gracilis* and *N. oceanica* at 8% in diets for seabass led to a reduction in several microbial diversity measures, but when these algae were included in a blend, it helped re-establish the lost diversity (Ferreira et al., 2022). One opinion is that a reduction in bacterial diversity can have negative impacts on fish health (Legrand et al., 2020). Based on our observations, the inclusion of algae blends in diets for European seabass does not have a negative impact on the overall intestinal microbial diversity.

Although significant differences were not noted for any of the microbial diversity measures, it was evident that the algae blend had an impact on specific bacterial groups but only at the 2% inclusion level. When compared to the Blend 2 group, fish in all other dietary treatments (CTRL, Blend 4, and Blend 6) showed a higher abundance of certain bacterial groups, including *Burkholderia*, *Haliscomenobacter*, *Neisseria*, *Streptococcus*, and *Vogesella*. Although the genera *Burkholderia* and *Streptococcus* encompass pathogenic species (Dawood, 2021; Legrand et al., 2020), it is worth noting that *Streptococcus* also hosts strains with probiotic effects (Swain et al., 2009). Additionally, the presence of *Burkholderia* in the intestine has been associated with improved health (Wang et al., 2018). Interestingly, previous studies have reported a decrease in both *Burkholderia* and *Streptococcus* in European seabass fed diets supplemented with extracts or algal powder derived from *G. gracilis* (Gonçalves et al., 2022). In this study, the identified ASVs categorized as *Burkholderia* showed close relationships with strains of *Burkholderia puraquae* and *Burkholderia contaminans*, both part of the *Burkholderia cepacia* complex, which is a well-known opportunistic human pathogen (Martina et al., 2018; Vanlaere et al., 2009). The *Streptococcus* ASVs modulated by the diets clustered with strains of *Streptococcus salivarius*, *Streptococcus sanguinis*, *Streptococcus pseudopneumoniae* and *Streptococcus mitis*. Among these, *S. salivarius* is known as a lactic acid-producing bacterium with recognized probiotic potential (Hwanhlem et al., 2011), while the other species are human commensals and potential opportunistic pathogens (Ikryannikova et al., 2013).

Increase in the abundance of the genus *Acinetobacter*, which includes both opportunistic pathogenic species (Legrand et al., 2020) and probiotic strains (Bunnoy et al., 2019), was observed in the CTRL and Blend 4-fed fish compared to the Blend 2 group. The ASVs modulated in this study were most likely related to *Acinetobacter radioresistens* and *Acinetobacter baumannii*. Both have been previously associated with severe infections in humans (Wang et al., 2019), and the latter has also been implicated in fish infections (Xia et al., 2008). The genus *Enterobacter* was more prevalent in the CTRL and Blend 6 groups in comparison with the fish that consumed Blend 2. The identified *Enterobacter* ASV was likely related to *Enterobacter cloacae*, a bacterium recognized for its probiotic activity against fish pathogens (Rajagopalan Girijakumari et al., 2018). However, it's important to note that strains of *E. cloacae* have also been linked to diseases in fish (Preena et al., 2021). Additionally, there was a significant increase in the representation of the genus *Corynebacterium*, comprising potentially pathogenic bacteria (Friberg et al., 2019), in fish consuming Blend 4 and Blend 6 diets.

Some alterations were specific to certain diets. For example, there were increases in *Brevundimonas* and *Rhizobium* in the CTRL fish, *Tepidimonas* in the Blend 4 group, and *Runella* in the Blend 6 group. These genera encompass potentially pathogenic bacteria, such as *Brevundimonas* (Zhou et al., 2021), bacteria with cellulolytic and pectolytic activity like *Rhizobium* (Xia et al., 2018), and bacteria associated with the production of alkaline protease and lipase, namely *Tepidimonas* (Wang et al., 2020). Furthermore, in the CTRL fish, a decreased representation of *Anoxybacillus* was observed. This anaerobic bacterium has been previously associated with the mucin-producing gene *muc13* (Naya-Català et al., 2021). Additionally, a *Plesiomonas* ASV, which clustered with strains of *Plesiomonas shigelloides*, an opportunistic pathogen that may cause disease in fish under environmental stressors, had lower abundance in these fish (Cortés-Sánchez et al., 2021).

In the present study, only the lowest inclusion level of the algae blend (2%) led to modulations in certain gut microbiota groups compared to the CTRL group. However, these findings did not entirely align with the changes in growth performance, gut morphology, and disease resistance. Interestingly, as the inclusion levels of the algae blend increased, we noted a corresponding increase in final body weight of the fish. Among the groups, the Blend 6-fed fish exhibited the highest final body weight, followed by the Blend 4 group (Mota et al., 2023). Coincidentally, the observed alterations in gut structure were predominantly evident in fish that consumed blends 4 and 6. In a complementary study, where we evaluated the *in vivo* immunomodulatory activities of the algae blend following a bath challenge with the pathogen *Tenacibaculum maritimum*, we could demonstrate the blend's ability to activate an effective mucosal immune response, rendering European seabass more capable of dealing with infections at the point of entry. Notably, these effects were

again most pronounced in the Blend 4 and Blend 6 groups. The 4% inclusion level, in particular, led to a more distinct immune modulation in comparison to the other blend-fed groups, accompanied by lower mortality (Ferreira et al., 2023b).

The findings from the present study collectively suggest that the tested commercial algae blend, ALGAESSENCE™ – Feed, significantly influences the intestinal morphology and microbial structure of European seabass. However, the extent of these effects appears to be closely linked to the incorporation levels. It is worthy to note that changes in certain bacterial groups did not seem to directly correspond to the changes observed in terms of intestinal morphology, growth performance, and immune responses. As a result, further research is warranted to fully elucidate the functional implications of these alterations. Future studies should consider the implementation of more advanced techniques for studying the gut microbiota, such as metagenomics and metatranscriptomics. This will allow for a comprehensive exploration of the potential of algae in modulating intestinal bacterial communities and their probable functions, which can be correlated to fish overall performance and health. Ultimately, this knowledge will be instrumental in developing refined functional aquafeeds capable of enhancing fish health and performance.

4.5 Data availability statement

The data used in this study is available at Sequence Read Archive (SRA) with accession number PRJNA1031113.

4.6 Author contributions

LMPV and VK were responsible for acquiring funding and conceptualizing the study. MF and YA conducted the formal analysis and investigation. HA and JS provided the necessary resources for the study. MF initially drafted the manuscript under the supervision of LMPV and VK, who subsequently reviewed and edited it. All authors have read, revised, and approved the final version of the manuscript.

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4.8 Acknowledgements

The support received from Bisa Saraswathy, Researcher, Nord University, for data analyses and preparation of the manuscript is acknowledged.

4.9 Conflict of interest

HA and JS were employed by the companies ALGAplus and Allmicroalgae, respectively. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Chapter 5.

Micro- and macroalgae blend modulates the mucosal and systemic immune responses of European seabass (*Dicentrarchus labrax*) upon infection with *Tenacibaculum maritimum*

By

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Abstract

Intensification of aquaculture practices is often associated with disease outbreaks. These outbreaks can heavily affect the production of several fish species such as European seabass (*Dicentrarchus labrax*) that is of high economic relevance in Mediterranean countries. Nutritional approaches are promising solutions to alleviate the burden caused by diseases. In this context, algae-derived bioactive compounds, which can stimulate fish's immune system, should be considered to elevate the health status of farmed fish.

Here we thoroughly evaluated the immunomodulatory activity of a commercially produced micro- (*Nannochloropsis oceanica* and *Chlorella vulgaris*) and macroalgae (*Gracilaria gracilis* and *Ulva rigida*) blend, both at the systemic and mucosal levels, focusing on the protective potential against *Tenacibaculum maritimum*, a pathogen heavily affecting farmed seabass. A commercial-based diet rich in plant proteins (CTRL) was compared against 3 experimental diets (blend 2, 4 and 6) with increasing levels of the algae blend (2, 4 and 6% inclusion, respectively). After a 12-weeks feeding trial, European seabass juveniles were subjected to a bath challenge with *T. maritimum* and relocated to two new systems: one system to collect tissues 48 h post-infection, and a second system to register mortalities over 8 days.

The results indicate that the algae blend is able to improve fish growth performance and evoke appropriate mucosal immune response upon infection with *T. maritimum*. The blend promoted a more exacerbated immune response, in a dose-dependent manner, by favoring the migration of monocytes and lymphocytes to mucosal tissues, which are the first sites to be affected by the pathogen. Such cellular responses were accompanied by an upregulation of genes associated with cell migration and proliferation (*mmp9* and *pcna*), and pro-inflammatory responses (*il-1 β* and *il-8*) in skin and gut tissues. At the systemic level, all blend diets elevated the plasma bactericidal activity and increased the expression of the antimicrobial peptide hepcidin in the head-kidney. The 4% incorporation level, but not the other levels, also led to a higher number of circulating peripheral neutrophils and acidic goblet cells in the intestine. Altogether, and although all blend diets improved fish growth and had some immunomodulatory effects, the 4% inclusion level seems the most suitable to prevent the adhesion and colonization of the pathogen on mucosal tissues, thereby inhibiting the progression of the disease and reducing mortality upon a challenge with *T. maritimum*. The present results highlight the importance of the mucosal immune response during bacterial infection and the potential of algae to modulate the mucosal immunity.

Keywords: Functional algae feeds; Tenacibaculosis; bath challenge; mucosal immunity; One Health

5.1 Introduction

Aquaculture, one of the most important food production sectors, is expected to meet the increasing food fish demand to satisfy the global population growth (Naylor et al., 2021). European seabass is a marine species of high economic value, and intensive farming of this species has caused disease outbreaks by Gram-negative bacteria (e.g., *Vibrio anguillarum*, *Photobacterium damsela* subsp. *piscicida* – Phdp, and *Tenacibaculum maritimum*) associated with high mortality rates and devastating economic losses (Fernández Sánchez et al., 2022). Fish farms still rely on antibiotics and chemotherapeutants to control bacterial outbreaks, a practice that not only contributes to the emergence of drug-resistant pathogens, but has also a negative connotation (Reverter et al., 2020). Innovative solutions that enhance fish immunity are required to control antimicrobial resistance, which is one of the most important public health threats, thereby contributing to the One Health concept (Cabello & Godfrey, 2019; Preena et al., 2020).

Fish overall performance and disease resistance is heavily dependent on adequate nutrition, and continuous efforts are being made to produce highly efficient diets supplemented with added-value products (Oliva-Teles, 2012). The protective effects of functional feeds for European seabass have been mainly explored in relation to *Vibrio* spp. and Phdp infections (Gisbert et al., 2018; Peixoto et al., 2019; Torrecillas et al., 2012). The few available reports on *T. maritimum* infection in farmed fish are centered around the characterization of the mucosal and systemic immune responses (Escribano et al., 2020; Frisch et al., 2018; Guardiola et al., 2019), with little information on the nutritional modulation to enhance resistance to this pathogen (Khalil et al., 2018). Moreover, effective commercial vaccines against tenacibaculosis are lacking, so dietary formulations capable of improving fish resistance to avoid high mortalities during a bacterial infection are a priority for the aquaculture industry.

Nutritional modulation that targets fish immunity has been explored for the past 40 years (Kiron, 2012), and more recently, the ability of algae-supplemented feeds to influence fish immune system and elevate the resistance to pathogens has caught the attention of the aquaculture sector. Both micro- and macroalgae are valuable sources of bioactive compounds with immunomodulatory effects, namely pigments, vitamins, minerals, and polysaccharides (Jacob-Lopes et al., 2019; Silva et al., 2020). Indeed, spirulina (*Arthrospira platensis*) supplemented diets led to an immunomodulation in European seabass, increasing survivability and fish resistance against *V. anguillarum* infection (Güroy et al., 2022). Messina et al. (2019) have also observed an increase in plasma lysozyme, antiprotease and myeloperoxidase activities in seabass offered a diet containing a blend of microalgae *Tisochrysis lutea* and *Tetraselmis suecica*. Likewise microalgae, the dietary

inclusion of *Laminaria digitata* and a commercial blend of seaweeds facilitated the establishment of a more efficient immune response in Atlantic salmon (*Salmo salar*) challenged with lipopolysaccharides; the seaweeds blend has altered more immune-related pathways compared with the single algae, which indicates an enhanced response when several algae are used simultaneously (Palstra et al., 2018).

Although most nutritional studies in fish are focused on the use of single algae species (Cerezuela et al., 2012b; Fumanal et al., 2020; Peixoto et al., 2019; Wassef et al., 2013), or in a combination of either microalgae or seaweeds (Messina et al., 2019; Palstra et al., 2018), the inclusion of two or more micro- and macroalgae may allow the activation of a synergetic immune modulation promoted by distinct species. A previous study *in vitro* with two microalgae (*Nannochloropsis oceanica* and *Chlorella vulgaris*), two macroalgae (*Gracilaria gracilis* and *Ulva rigida*) and a commercial blend of the four algae (Algaessence™ – Feed) reported an added-value antimicrobial potential of the blend compared to the single algae. Moreover, the highest *in vitro* bactericidal activity (45%) was observed against the pathogen *T. maritimum* (Ferreira et al., 2021). Hence, in the present study, we evaluated the *in vivo* immunomodulatory effects of the algae blend, i.e., Algaessence™ – Feed, on the systemic and mucosal immune system of European seabass fed plant-protein rich diets, focusing on the protective potential against tenacibaculosis.

5.2 Materials and methods

5.2.1 Ethical statement

The experimental trials with animals were performed by scientists accredited for conducting laboratory studies with animals, following the European Union guidelines (Directive 2010/63/EU). Furthermore, the protocols were approved by CIIMAR's (Centro Interdisciplinar de Investigação Marinha e Ambiental) animal welfare body (ORBEA-CIIMAR: 0421/000/000/2020) and by the Portuguese Veterinary Authority.

5.2.2 Experimental diets

A commercial algae blend (ALGAESSENCE™ – Feed) composed of two microalgae (*Nannochloropsis oceanica* and *Chlorella vulgaris*) and two macroalgae (*Gracilaria gracilis* and *Ulva rigida*) was produced at industrial scale by two companies in Portugal, Allmicroalgae (Pataias, Portugal) and ALGAplus (Ílhavo, Portugal). Four isonitrogenous (53% dry matter, DM) and isoenergetic (22 kJ g⁻¹ DM) diets were formulated and extruded by Sparos Lda., Olhão, Portugal. A control diet (CTRL) was formulated considering current tendencies in commercial aquafeeds, i.e., high levels of vegetable protein sources (71.8%) and moderate levels of fish meal (12.5%). The three experimental diets had increasing

levels (i.e., 2, 4 and 6%) of a commercial algae blend (blend 2, 4 and 6, respectively) included at the expense of whole peas and wheat gluten. The ingredient composition (%) and proximate analysis (% dry matter, DM) of the experimental diets is presented in Figure 1. The detailed chemical composition of the algae blend can be found in Ferreira et al. (2021).

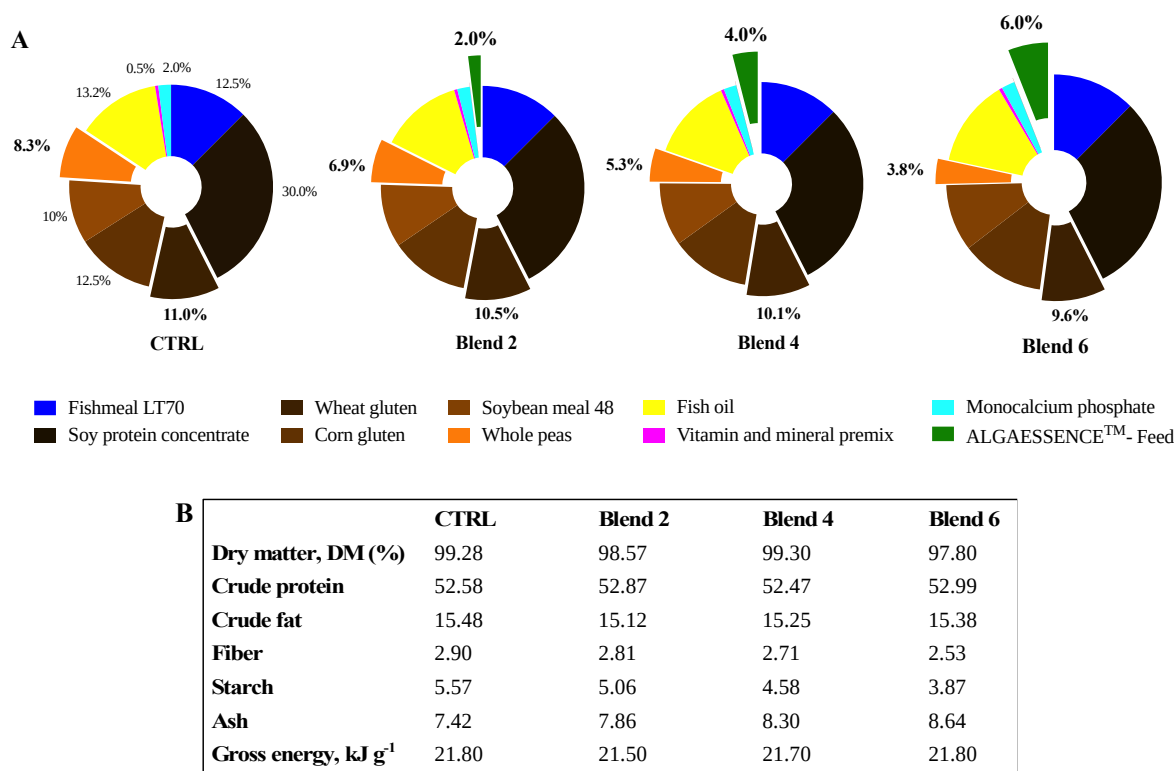


Figure 1. Composition of the experimental diets fed to European seabass 12 weeks prior to *T. maritimum* bath challenge. A: Ingredient composition (%) and B: proximate analysis (% dry matter, DM) of the experimental diets.

5.2.3 Growth trial

The growth trial was conducted in the animal facility of CIIMAR in Matosinhos, Portugal. European seabass juveniles were obtained from a commercial fish farm (ACUINUGA, Spain), and upon arrival the fish were kept in quarantine for 3 weeks and fed a commercial diet from Sorgal S.A., Portugal (49% crude protein and 20% crude fat, DM basis). Following acclimation and after a 24 h fasting period, fish were anesthetized with 0.5 mL L⁻¹ of 2-phenoxyethanol (Sigma-Aldrich, Germany) and individual weights were registered. Twelve homogeneous groups of 46 fish (average body weight 11.3 ± 2.7 g) were randomly distributed into 12 fiberglass tanks (250 L) within a saltwater recirculation system. The system temperature was maintained at 20.9 ± 0.4 °C, and salinity and pH were 35 ± 1.3‰ and 7.5 ± 0.16, respectively. Throughout the experiment we adhered to a 12 h light/12 h

dark photoperiod regime. Water quality parameters (i.e., ammonia, oxygen, redox potential, temperature, pH, and salinity) were monitored during the trial and the values remained stable for the feeding period. The experimental diets were randomly allocated to each tank, with triplicate tanks per diet. Fish were fed by automatic feeders, three times a day to apparent satiation, for 12 weeks (84 days). At the end of the trial, all fish were individually weighed, and samples of interest were collected for further analyses. The growth performance calculations were performed as follows: Weight gain = final body weight (FBW) – initial body weight (IBW); Specific growth rate (SGR) = $(\ln \text{FBW} - \ln \text{IBW}) / \text{days of the experiment} \times 100$.

5.2.4 Bacterial challenge and fish sampling

At the end of the growth trial, 50 fish per treatment (16-17 fish per tank) were subjected to a bath challenge with *Tenacibaculum maritimum* and redistributed into two new recirculating systems, with similar rearing conditions to those selected for the feeding trial. In the first system, duplicate groups of 10 fish per tank ($n = 20$ per treatment) were used for a 48-h challenge study, whereas triplicate groups of 10 fish per tank ($n = 30$ fish per treatment) were included to assess cumulative mortality. In the second system, and aiming to avoid horizontal infections, duplicate groups of 10 fish per tank that were submitted to similar handling (i.e., bath challenged with marine broth) but not infected with the bacterium were regarded as non-infected groups.

For the bacterial challenge it was employed a *T. maritimum* strain ACC13.1 isolated from Senegalese sole (*Solea senegalensis*) collected from a local fish farm (Portugal). The bacterial strain kindly provided by Professor Alicia E. Toranzo (University of Santiago de Compostela, Spain) was cultured in marine agar at 22 °C for 48 h prior to inoculation in marine broth for 18 h. To prepare the inoculum, exponentially growing bacteria were collected and adjusted to a final concentration of 3.4×10^5 colony forming units (cfu) L⁻¹. To carry out both the 48-h challenge and cumulative mortality studies, experimental procedures followed those carried out by Resende et al. (2022). Dead and moribund fish were analyzed to re-isolate the pathogen. Humane endpoints were considered for moribund fish and those with ulcerative lesions or necrotized skin (Mabrok et al., 2016). The amount of the bacterial strain that was able to kill 50% of the challenged fish (LD₅₀) was calculated according to the method of Reed and Muench (1938) prior to performing the challenge.

After the 48-h challenge study, five fish per tank from both infected and non-infected groups ($n = 10$ fish per treatment) were anesthetized with 0.5 mL L⁻¹ of 2-phenoxyethanol, and samples were collected. Blood was collected from the caudal vein of each fish for haematological analysis, and to obtain plasma after centrifuging at $10,000 \times g$ 10 min at 4

°C. Plasma samples were immediately deep-frozen and stored at -80 °C until the innate humoral immune response parameters were assessed. Small portions of skin, posterior intestine (before the ileo-rectal valve) and head-kidney tissues were sampled, preserved in RNAlater™ (Thermo Fisher Scientific, USA), and then transferred to 4 °C for 24 hours, before being stored at -80 °C until gene expression analysis. For intestinal histological evaluation, 0.5 cm of the posterior intestine was dissected out, washed, and fixed in formaldehyde (3,7-4%, pH 7) for 24 h, and then preserved in ethanol 70%.

For the long-term challenge, mortalities were followed and registered over a period of 8 days. The survivors at the end of the experiment were euthanized by immersion in 1 mL L⁻¹ of 2-phenoxyethanol and examined for the visual detection of *T. maritimum*.

5.2.5 Haematological profile

The haematological parameters, namely haematocrit (Ht), haemoglobin (Hb; SPINREACT kit, ref. 1001230, Spain) and total red (RBC) and white (WBC) blood cells counts, were determined in both non-infected and infected groups, following the protocols of Machado et al. (2015). The mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were then calculated as follows: $MCV (\mu m^3) = (Ht/RBC) \times 10$; $MCH (pg \text{ cell}^{-1}) = (Hb/RBC) \times 10$; $MCHC (g \text{ 100 mL}^{-1}) = (Hb/Ht) \times 100$.

To assess the differential blood count, blood smears were prepared and air dried, immediately after blood collection. Following fixation with formaldehyde-ethanol (10% of 37% formaldehyde in absolute ethanol), peroxidase was detected according to Afonso et al. (1998). Thereafter, the slides were stained with Wright's stain (Haemacolor; Merck, Germany) to examine (1,000 ×) a minimum of 200 leucocytes to classify thrombocytes, lymphocytes, monocytes, and neutrophils. The relative percentage of each leucocyte cell was used to calculate the absolute value ($\times 10^4 \mu L^{-1}$), based on the total WBC count (Machado et al., 2015).

5.2.6 Innate immune parameters in plasma

Plasma bactericidal activity, lysozyme, peroxidase, and alternative complement pathways (ACH50) activities were measured in both non-infected and infected groups, in triplicate and using a microplate spectrophotometer (BioTek Synergy HT, USA).

Plasma bactericidal activity was determined according to Graham et al. (1988) and Sunyer and Tort (1995), with modifications. *T. maritimum* was grown in marine agar (Condalab, Spain) and resuspended in marine broth (MB; Condalab, Spain). The bacterial suspensions were adjusted to Absorbance (Abs) values of 0.3 at Abs.₆₀₀, corresponding to 3×10^8 cfu

mL⁻¹. Plasma samples (20 µL) were incubated with 20 µL of bacterial suspension in U-shaped 96-well microplate on a shaker, for 2.5 h at 25 °C. The samples were, thereafter, incubated with 25 µL of thiazolyl blue tetrazolium bromide (Sigma-Aldrich, Germany) at 1 mg mL⁻¹ on a shaker, for 10 min at 25 °C, and then centrifuged for 10 min at 2,000 × g. The supernatant was discarded, and the obtained pellet was dissolved in 200 µL dimethyl sulfoxide (DMSO, Sigma-Aldrich, Germany). After complete dissolution, 100 µL of each sample were placed into a flat-bottomed 96-well microplate and the Abs.₅₆₀ was measured. The bactericidal activity was calculated according to the percentage of non-viable bacteria, using the following formulae: % viable bacteria = [(Abs sample) / (Abs positive control)] × 100; % non-viable bacteria = 100 - % viable bacteria.

Lysozyme activity was estimated using a turbidimetric assay adapted to microtiter, as described by Costas et al. (2011). Briefly, 15 µL of each sample were added to 250 µL of a *Micrococcus lysodeikticus* (Sigma-Aldrich, Germany) solution (0.5 mg mL⁻¹, 0.05 M sodium phosphate buffer, pH 6.2) in a flat-bottomed 96-well plate. The Abs.₄₅₀ was measured at 0, 10, 15 and 20 min. A standard curve was prepared using serial dilutions of lyophilized hen egg white lysozyme (Sigma-Aldrich, Germany) in sodium phosphate buffer and used to calculate the lysozyme activity of each sample.

Peroxidase activity was determined according to the assay described by Quade and Roth (1997). Briefly, 7.5 µL of plasma were added to 142.5 µL of HBSS without Ca²⁺ and Mg²⁺ in a flat-bottomed 96-well plate. Tetramethylbenzidine hydrochloride (50 µL at 10 mM; Sigma-Aldrich, Germany) was then added, followed by immediate addition of 50 µL of 5 mM H₂O₂. After 2 min, 50 µL of 2 M sulfuric acid were added to stop the reaction. At the end of the assay, the Abs.₄₅₀ was measured and the peroxidase activity (units mL⁻¹ plasma) was determined as follows: one unit of peroxidase produces an absorbance change of 1 OD.

ACH50 was evaluated according to Sunyer and Tort (1995). Briefly, two solutions were prepared: a test solution at pH 7.3, containing 0.1% gelatin, 5 mM sodium barbiturate, 0.13 mM sodium chloride, 10 mM Mg²⁺ and 10 mM glycol ether diamine tetraacetic acid (EGTA); and a stop solution at pH 7.3, containing 0.1% gelatin, 5 mM sodium barbiturate, 0.13 mM sodium chloride and 20 mM ethylenediamine tetraacetic acid (EDTA). Rabbit red blood cells (RaRBC; Probiologica Lda., Portugal) were then washed at least four times in sodium chloride solution 0.9%, and later diluted in the same solution to a final concentration of 2.5 × 10⁸ cells mL⁻¹. The RaRBC suspension (10 µL) was then added to 40 µL of serially diluted plasma in test solution in a U-shaped 96-well microplate. The plates were incubated for 100 min at room temperature with continuous shaking and the reaction was stopped after the addition of 150 µL of stop solution. Following a 2.5 min centrifugation at 120 × g, the supernatant was transferred to flat-bottomed 96-well plates and the Abs.₄₁₄ was measured.

The ACH50 units were defined as the concentration of plasma resulting in 50% haemolysis of RaRBC.

5.2.7 Expression of immune-related genes in head-kidney, skin, and posterior intestine

Total RNA was extracted from head-kidney, skin, and posterior intestine samples (~15 mg), as described by Ferreira et al. (2020). Quantity and purity of the extracted RNA were evaluated by spectrophotometry (A260/A280 ratios), using DeNovix DS-11 Series Spectrophotometer (DeNovix Inc., USA). To evaluate RNA integrity, samples were loaded into a 1% TAE (w/v; NZYTech, Portugal) agarose electrophoresis gel stained with GreenSafe Premium (NZYTech, Portugal). First-strand cDNA was then synthesized using a NZY First-Strand cDNA Synthesis Kit (Nyztech, Portugal), with 1 µg of RNA as starting material in a final volume of 20 µL. Real-time PCR (RT-PCR) assays were performed on a CFX384 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, USA) with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, USA). Reactions were carried out in duplicate with 400 nM of each primer (forward and reverse), 5 µL of Green Supermix and 2 µL of cDNA (diluted 1:10), in a total reaction volume of 10 µL, with the following thermal cycling conditions: 95 °C for 30 sec, followed by 35 cycles of two steps of 95 °C for 5 sec and 60 °C for 30 sec. A negative control without cDNA was included to confirm that the reagents were free of contaminants. To ensure reaction specificity, a post-amplification dissociation curve (65–95, 0.5 °C increments at 2-5 sec/step) was performed at the end of each run. PCR efficiency was obtained for each gene (four reference genes and 14 targeted immune-related gene), using a 5-fold serial dilution of cDNA from the samples from all experiments. The immune-related genes included pro- (i.e., *il-1β*, *il-8* and *tnf-α*) and anti-inflammatory (i.e., *il-10*) cytokines/chemokines, toll-like receptor 9 (*tlr9*), caspases 3 and 9 (*casp3* and *casp9*), matrix metalloproteinase 9 (*mmp9*), the subunit P65 of the transcription factor nuclear factor-κB (*p65*), the macrophage colony-stimulating factor receptor (*mcsfr*), the proliferating cell nuclear antigen (*pcna*), and the antimicrobial peptides dicentracin (*dic*), piscidine 1 (*pisc1*) and hepcidin (*hep*). The primers, specific annealing temperatures and PCR efficiencies are described in Table 1. The geNorm algorithm (Vandesompele et al., 2002), implemented in qbase + software, version 3.2 (Biogazelle, Zwijnaarde, Belgium - www.qbaseplus.com), was used to determine the stability of the four selected reference genes (*18s*, *β-actin*, *ef1α* and *gapdh*). The three most stable genes in each tissue (*ef1α*, *β-actin* and *18s* for skin and head-kidney samples; *β-actin*, *ef1α* and *gapdh* for posterior intestine samples) were used to calculate a gene expression normalization factor for each sample. The relative abundance of the target genes was

calculated based on the comparative critical threshold ($\Delta\Delta CT$) method (Livak & Schmittgen, 2001), and the average CT for a given gene was used to transform the CT of each sample to relative quantities that were then normalized using the factor calculated for each sample using the reference genes.

5.2.1 Histology of posterior intestine

Samples preserved in 70% ethanol were processed following standard histological procedures and then embedded in paraffin. Cross sections of the posterior intestine (3 μm) samples were obtained using a semi-automated rotary microtome (Leica RM 2245). These sections were then stained with Alcian blue/Periodic Acid Schiff (AB/PAS, pH 2.5) or haematoxylin-eosin (HE). One cross-section from each fish was examined using a light microscope (Olympus BX51, Germany) with a camera (Olympus DP50). Using an imaging software (cell[^]B software from Olympus), the average number of goblet cells (GCs), specifically acid GCs (blue) and neutral GCs (pink), was determined by manual counting in 8 of the longest villi in each section (AB/PAS-stained section). The HE stained section was used to perform a semi-quantitative histological assessment of the mucosal fold length, submucosa width and lamina propria width as described by Silva et al. (2015).

5.2.1 Statistical analysis

The results of haematological and innate immune parameters, and immune-related gene expression are presented as fold change based on the values of the infected and non-infected groups. Statistical analyses were performed using SPSS 26.0 (SPSS Inc., Chicago, IL, USA). Data were checked for normality and homogeneity of variances with Kolmogorov-Smirnov and Levene's tests, respectively, and transformed whenever necessary. If normality and/or homogeneity of variances assumptions were not met after transformation, a non-parametric test (Kruskal-Wallis H-test) was used. To compare individual means, one-way ANOVA followed by Tukey's multiple comparison test was performed. Differences in the cumulative mortality of the study groups were identified using the Chi-square test. For all statistical analysis, the level of significance used was $P < 0.05$. The principal component analysis (PCA) was applied to all variables with significant differences using XLSTAT version 2022.1.2. (Addinsoft, USA).

Table 1. Target and reference genes and respective primers used in the reverse transcription-qPCR analysis performed using the head-kidney, skin, and posterior intestine tissues. The annealing temperature (°C), PCR efficiency (%) for each tissue, GenBank accession number and reference from where the primers were retrieved are presented.

Gene	Primer Forward	Primer Reverse	Annealing Temperature (°C)	Efficiency (%)			GenBank accession number	Reference
				Head-Kidney	Skin	Intestine		
<i>18s</i>	CCAACGAGCTGCTGACC	CCGTTACCCGTGGTCC	60	99	109	109	AY831388	Núñez-Ortiz et al. (2016)
<i>βactin</i>	CAAAGCCAACAGGGAGAAGATGA	ACCGGAGTCCATGACGATAC	60	96	104	108	AJ537421	Couto et al. (2015)
<i>ef1α</i>	AAC TTCAACGCCAGGTCAT	CTTCTTGCCAGAACGACGGT	60	101	107	105	AJ866727	Azeredo et al. (2017a)
<i>gapdh</i>	CAAGGTTGAGGGTGACAAGC	CAGCCTCACCCCATTTGATG	60	-	107	105	AY863148	Couto et al. (2015)
<i>il-1β</i>	AGCGACATGGTGCGATTCT	CTCCTCTGCTGTGCTGATGT	60	103	100	105	AJ269472	Azeredo et al. (2017b)
<i>il-8</i>	CGCTGCATCCAAACAGAGAGCAAAC	TCGGGGTCCAGGCAAACCTCTT	60	99	104	107	AM490063	Machado et al. (2018)
<i>tnf-α</i>	AGCCACAGGATCTGGAGCTA	GTCCGCTTCTGTAGCTGTCC	60	107	96	100	DQ070246	Machado et al. (2018)
<i>il-10</i>	ACCCCGTTCGCTTGCCA	CATCTGGTGACATCACTC	60	97	99	92	AM268529	Machado et al. (2021)
<i>tlr9</i>	TCTTGTTTGCCGACTTCTGCGT	TACTGTTGCCCTGTTGGGACTCTGG	60	-	103	100	KX399289	Machado et al. (2018)
<i>casp3</i>	TGATGTCGTCTCTGCCGTAG	ACCACCTCATACGCATCCTC	60	-	108	106	DQ345773	Couto et al. (2015)
<i>casp9</i>	GGCAGGACTCGACGAGATAG	CTCGCTCTGAGGAGCAAAC	60	98	108	105	DQ345775	Sepulcre et al. (2007)
<i>pcna</i>	TGAAGTGTGCAGGAAACGAAGA	GGCGAGTGTGTCTGCATTGT	60	103	106	104	JQ755266	Rawling et al. (2019)
<i>mmp9</i>	TGTGCCACCACAGACAACCTT	TTCCATCTCCACGTCCCTCA	60	96	97	104	FN908863	Machado et al. (2018)
<i>mcsfr1</i>	ATGTCCCAACCAGACTTTGC	GGCTCATCACACACTTCACC	60	107	108	101	DLAgn_00109630	-
<i>p65</i>	GTGTGGTTGTGTTGCCTTG	CCCTGAACCATCTCGACTA	60	107	107	107	DLAgn_00141590	Machado et al. (2021)
<i>dic</i>	TGCGCCACGCTCTTTCTT	CCCCAGGTTAGCCATGAG	60	-	105	100	AY303949	Terova et al. (2011)
<i>pisc1</i>	TCGTCCTCATGGCTGAACC	GCGGTTAAAGCGTGATATTG	60	-	102	103	MT066191	Barroso et al. (2020)
<i>hep</i>	CTGGAGGAGCCAATGAGCAA	TGGAGAGAGCATCAGAGCAC	60	97	102	94	DQ131605	Machado et al. (2018)

18S, 18S rRNA; *βactin*, Beta actin; *ef1α*, Elongation factor 1 alpha; *gapdh*, Glyceraldehyde-3-phosphate dehydrogenase; *il-1β*, Interleukin-1 beta; *il-8*, Interleukin-8; *tnf-α*, Tumor necrosis factor-alpha; *il-10*, Interleukin-10; *tlr9*, Toll-like receptor 9; *casp3*, Caspase 3; *casp9*, Caspase 9; *pcna*, Proliferating cell nuclear antigen; *mmp9*, Matrix-metalloproteinase 9; *mcsfr1*, Macrophage colony-stimulating factor receptor 1; *p65*, Nuclear factor NF-kappa-B p65 subunit; *dic*, Dicentracin; *pisc1*, Piscidine 1; *hep*, Hepcidin precursor

5.3 Results

5.3.1 Growth performance

After the 12-weeks feeding trial, European seabass fed diets with the algae blend had a significantly higher final body weight, weight gain and specific growth rate (SGR) compared to those consuming the CTRL diet. The final body weight of fish fed the blend 6 was also significantly higher than those of seabass fed the other blend diets (Figure 2).

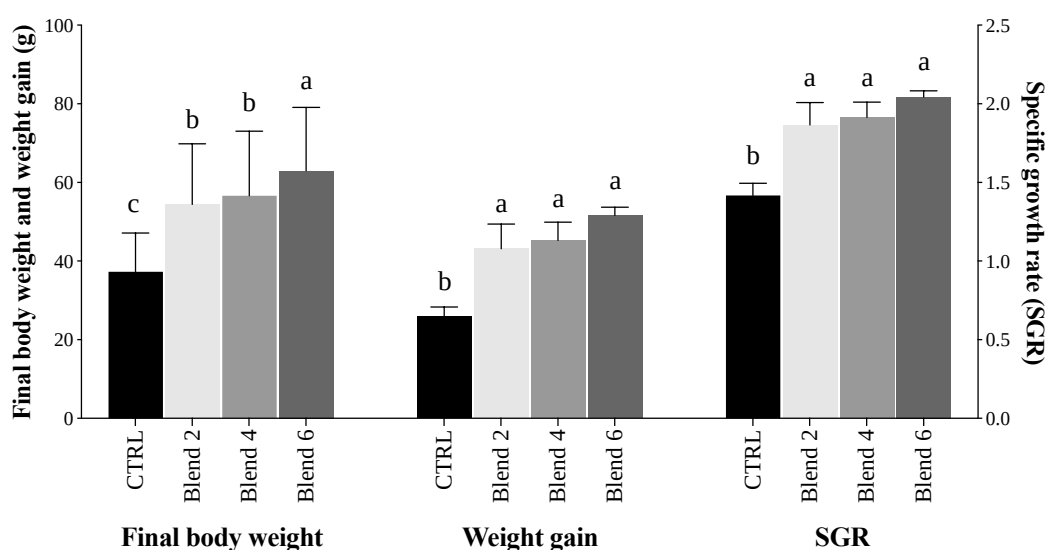


Figure 2. Final body weight, weight gain and specific growth rate (SGR) of European seabass fed the experimental diets for 12-weeks. Results are displayed as mean $\pm$ SD and different letters stand for significant differences among dietary treatments. For final body weight, $n = 40$ per treatment was considered (individual weights measured); while for weight gain and SGR, $n = 3$ per treatment was used, with n value representing average weight per tank.

5.3.2 Haematological profile

The haematological profile of European seabass fed diets with increasing inclusion levels of the algae blend for 12 weeks, and subsequently subjected to a bacterial bath challenge (*T. maritimum*), was evaluated. At 48 h post-infection, the haemoglobin, haematocrit, MCV, MCH and MCHC remained unaffected by the experimental diets (Figure 3A). RBC were significantly higher in fish fed CTRL compared to those consuming the blend 4 diet, while WBC were also highest in CTRL fish but only differing significantly from fish fed blend 6 (Figure 3A). The number of thrombocytes was significantly higher in fish either fed CTRL or blend 4 diets compared to blend 6 fish (Figure 3B). The experimental diets had also significant effects on the number of peripheral blood leucocytes (i.e., lymphocytes, monocytes, and neutrophils). The CTRL fish presented a significantly higher number of lymphocytes in comparison with

blends 2 and 4 fish, and an increased number of monocytes compared to all blend diets. The number of neutrophils was higher in fish consuming the blend 4 diet compared to all other diets (Figure 3B).

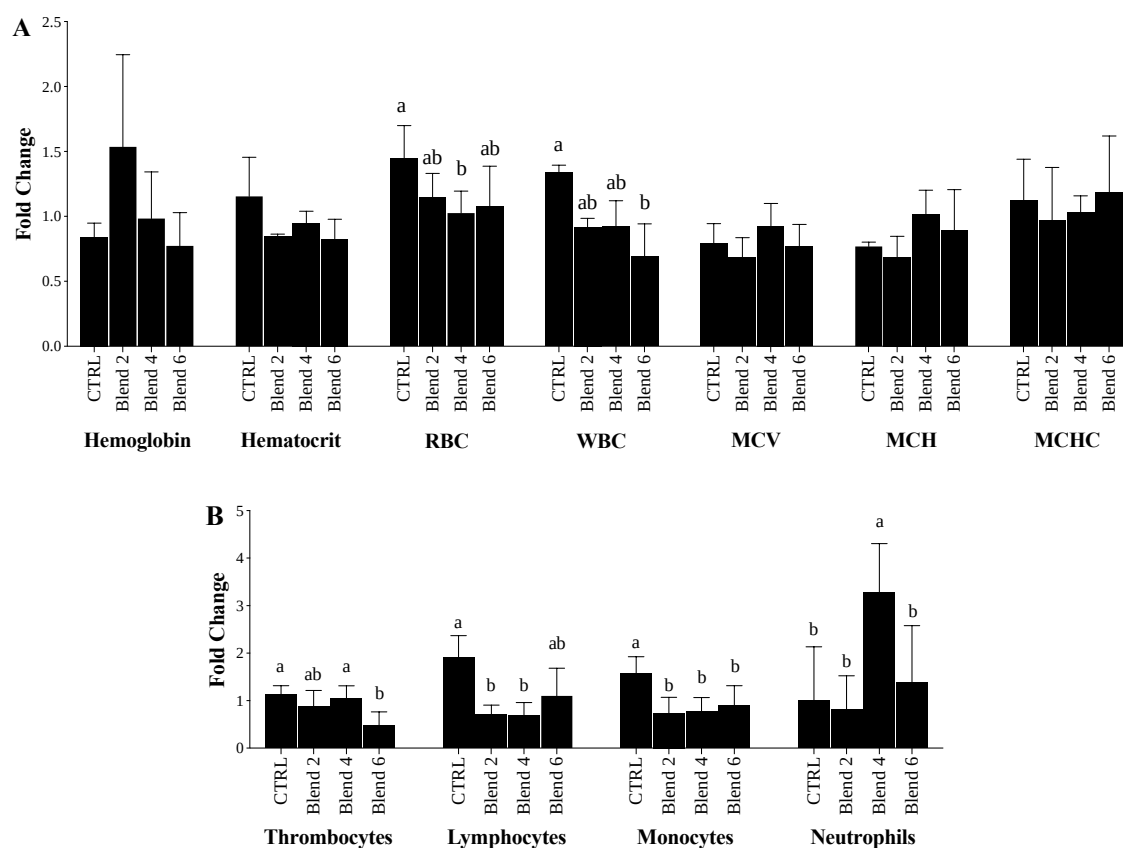


Figure 3. Haematological profile of European seabass fed the experimental diets for 12 weeks and subsequently subjected to *T. maritimum* bath challenge. A: Haemoglobin, haematocrit, red blood cells (RBC), white blood cells (WBC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC). B: Differential blood cells counting - thrombocytes, lymphocytes, monocytes, and neutrophils. Values are presented as fold change based on the values of the infected and non-infected groups, and results are displayed as mean $\pm$ SD (n = 6). Different letters stand for significant differences among dietary treatments.

5.3.3 Innate immune parameters in plasma

The effects of the experimental diets on the innate immune parameters are presented in Figure 4. The dietary treatments had no impact on ACH50 and peroxidase activity. Fish fed the blend diets had a significantly higher plasma bactericidal activity compared to the CTRL fish, with the highest value being reached in fish fed the blend 6 diet. On the other hand, the lysozyme activity was significantly higher in fish that consumed the CTRL diet compared to the fish fed the blend 6 diet.

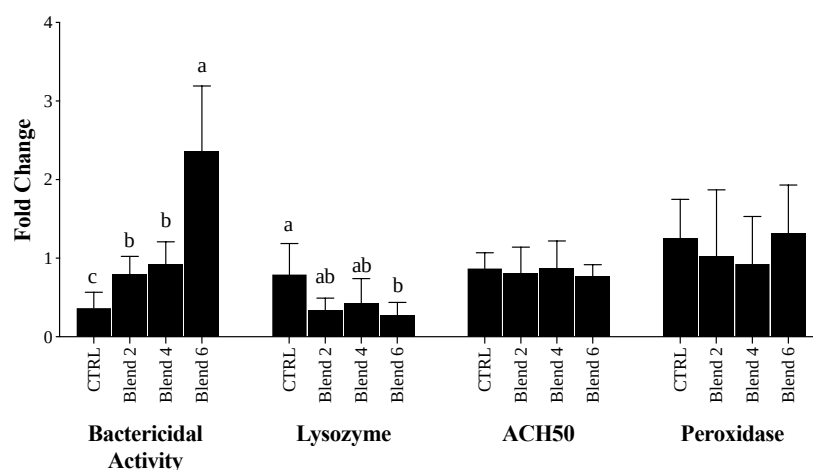


Figure 4. Innate immune parameters of European seabass fed the experimental diets for 12 weeks and subsequently subjected to *T. maritimum* bath challenge. ACH50: alternative complement pathways activity. Values are presented as fold change based on the values of the infected groups and non-infected groups, and results are displayed as mean $\pm$ SD (n = 10). Different letters stand for significant differences among dietary treatments.

5.3.4 Expression of immune-related genes in head-kidney, skin, and posterior intestine

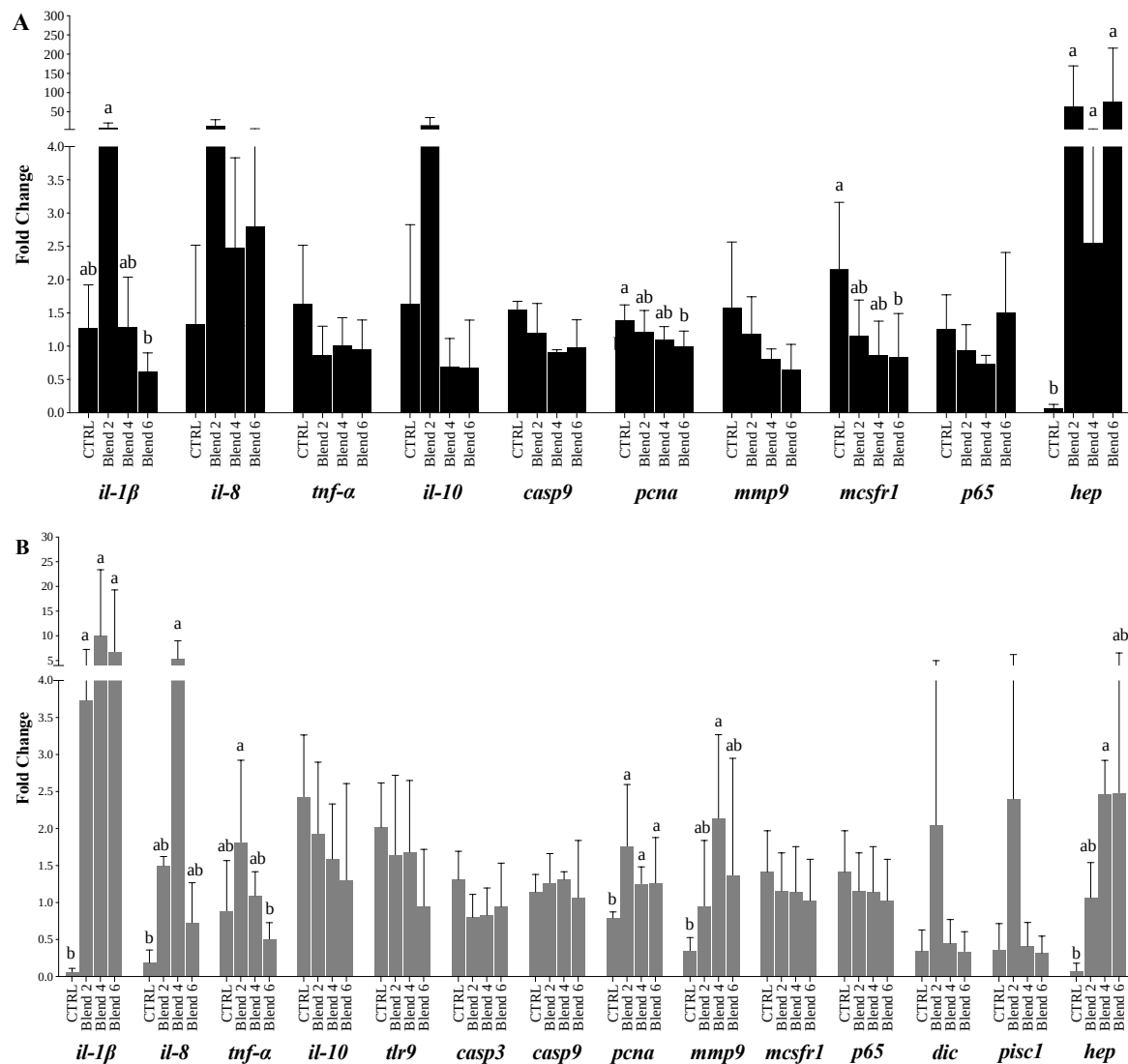
Expression of a panel of 14 genes that are reported to be involved in different immune pathways and response against pathogens were analyzed in the head-kidney, skin, and posterior intestine of European seabass fed the experimental diets and subsequently subjected to a bacterial bath challenge with *T. maritimum*.

Regarding the transcript levels at the head-kidney level, the *il-1 β* was upregulated in fish fed blend 2 diet, but only compared to those fed blend 6. Expression of *mcsfr* and *pcna* increased in CTRL fish compared to the fish fed blend 6, while the *hep* gene was significantly upregulated in fish fed blend diets compared to CTRL fish (Figure 5A).

The dietary treatments had significant impacts on several immune genes of both the skin and posterior intestine tissues (Figure 5B-C). The expression of *il-1 β* gene was higher in the skin of all fish fed the blend diets compared to the CTRL. On the other hand, *il-8* increased only in the blend 4 fed fish compared to the CTRL fish. Fish fed blend 2 had higher expression of *tnf- α* but only compared to the blend 6 fed fish. All fish fed the blend diets had a higher expression of *pcna* compared to the CTRL fish, irrespectively of the algae inclusion level. A significant upregulation of *mmp9* and *hep* was also observed in fish that consumed the blend 4 diet compared to the fish fed the CTRL diet (Figure 5B).

Concerning the posterior intestine, *il-1 β* and *il-8* were significantly upregulated in fish fed blend 2 and 4 diets, compared to the CTRL fish, while *tnf- α* expression was only upregulated in fish fed blend 4. The expression of *casp9* increased in the posterior intestine of the fish fed blend

6 compared to those fed other blend levels, without differing from the CTRL group. As observed in the skin, the fish fed the blend diets had also a higher expression of *pcna* in the intestine compared to the CTRL fish. The gene *mmp9* was significantly upregulated in the fish that consumed the blend 4 and blend 6 diets compared to other diets. Regarding the antimicrobial peptides *pisc1* and *hep*, while a higher expression of *pisc1* was observed in the CTRL fish compared to the blend 2 fed fish, an upregulation of *hep* was observed in the fish fed the blends 2 and 4 diets compared to the CTRL fish (Figure 5C).



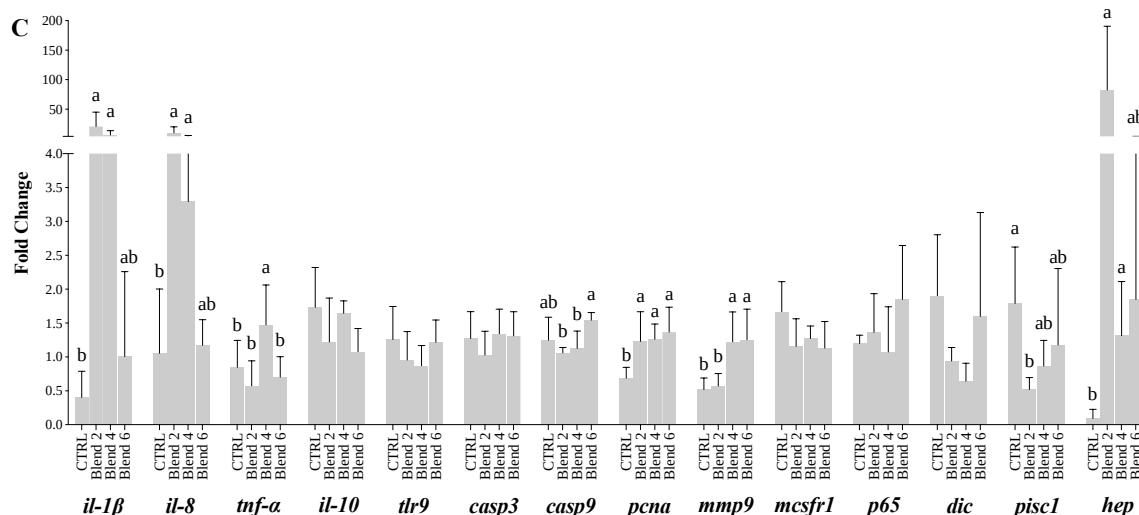


Figure 5. Expression of immune-related genes in the head-kidney (A), skin (B), and posterior intestine (C) of European seabass fed the experimental diets for 12 weeks and subsequently subjected to *T. maritimum* bath challenge. Values are presented as fold change based on the values of the infected and non-infected groups, and results are displayed as mean $\pm$ SD (n = 8). Different letters stand for significant differences among dietary treatments.

5.3.5 Histomorphological analysis of the posterior intestine

The histomorphological changes observed in the posterior intestine of European seabass fed diets with increasing levels of the algae blend for 12 weeks and subjected to a bacterial challenge with *T. maritimum*, are presented in Figure 6. The total number GCs and acid GCs were significantly higher in the intestine of the fish that consumed the blend 4 diet compared to fish fed the CTRL diet. The experimental diets did not significantly alter the neutral GC counts, mucosal folds length and submucosa and lamina propria widths.

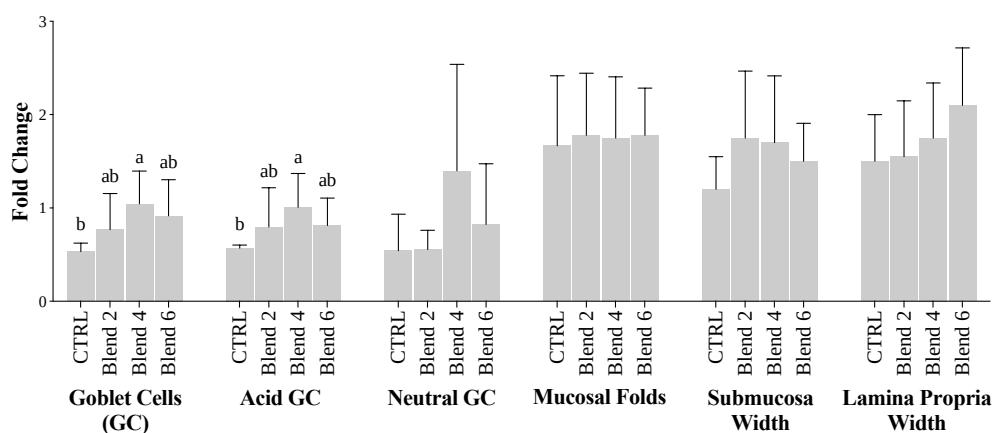


Figure 6. Histomorphological changes observed in the posterior intestine of European seabass fed the experimental diets for 12 weeks and subsequently subjected to *T. maritimum* bath challenge. Values are presented as fold change based on the values of the infected and non-infected groups, and results

are displayed as mean $\pm$ SD (n = 10). Different letters stand for significant differences among dietary treatments.

5.3.6 Cumulative mortality

To further understand the possible protective effects of diets with the algae blend inclusion during a bacterial challenge, fish were subjected to a bath challenge with *T. maritimum*, and their mortality was registered for 8 days. Mortalities were observed in all groups already at 24 h following bath infection, peaking at 48 h, and no mortalities were observed following 4 days after infection. European seabass fed blends 4 and 6 had the lowest cumulative mortalities (33 and 50%, respectively), followed by fish fed the CTRL diet (53%). Significant differences were only found between blend 4 (33%) and blend 2 (63%) (Figure 7).

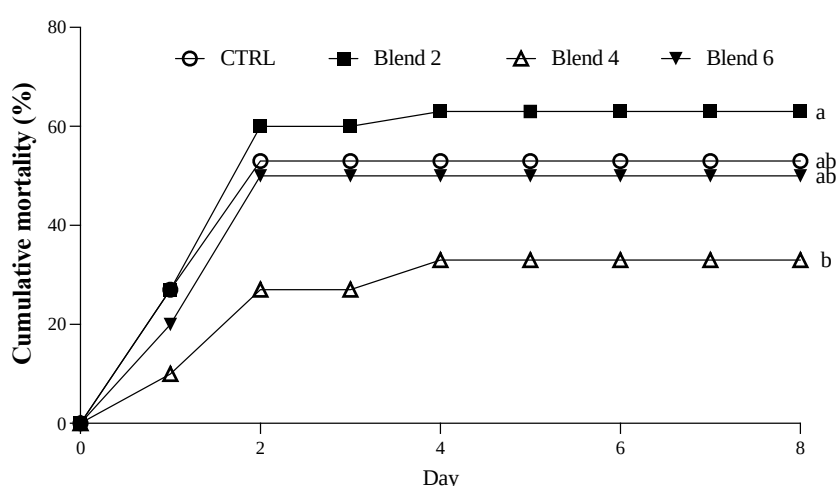


Figure 7. Cumulative mortality (%) of European seabass fed the experimental diets for 12 weeks and subsequently subjected to *T. maritimum* bath challenge. Different letters denote significant differences among treatments ($\chi^2 = 0.02$) and n = 30.

5.3.7 Principal component analysis

Dimensionally reduction employing principal component analysis (PCA) allowed us to summarize and visualize the key differentially regulated variables that contribute to the majority of the variance observed in the dataset. The first two dimensions of the PCA biplot explain 42.02% of the variability of the experimental data (Figure 8). Fish fed the blend 4 and blend 6 diets are grouped together and discriminated from fish that consumed the CTRL and blend 2 diets. Variables related with haematological profile, namely WBC, RBC, thrombocytes, lymphocytes, monocytes, and neutrophils, as well the head-kidney genes *mcsfr* and *pcna*, appeared in the quadrant were most of the CTRL fish clustered in the PCA plot. The head-kidney *il-1 β* , skin *pcna* and *tnf- α* , and posterior intestine *hep*, *il-8*, *il-1 β* were located on the F1

positive/F2 negative quadrant along the 95% confidence ellipse of the blend 2 fed fish. The fish that consumed blends 4 and 6 diets were grouped in the F2 positive quadrant and the associated variables were immune genes expressed in skin (*hep*, *il-8*, *il-1 β* , *mmp9*) and posterior intestine (*mmp9*, *casp9* and *tnf- α*), the intestine GC and acid GC numbers, and the blood neutrophils.

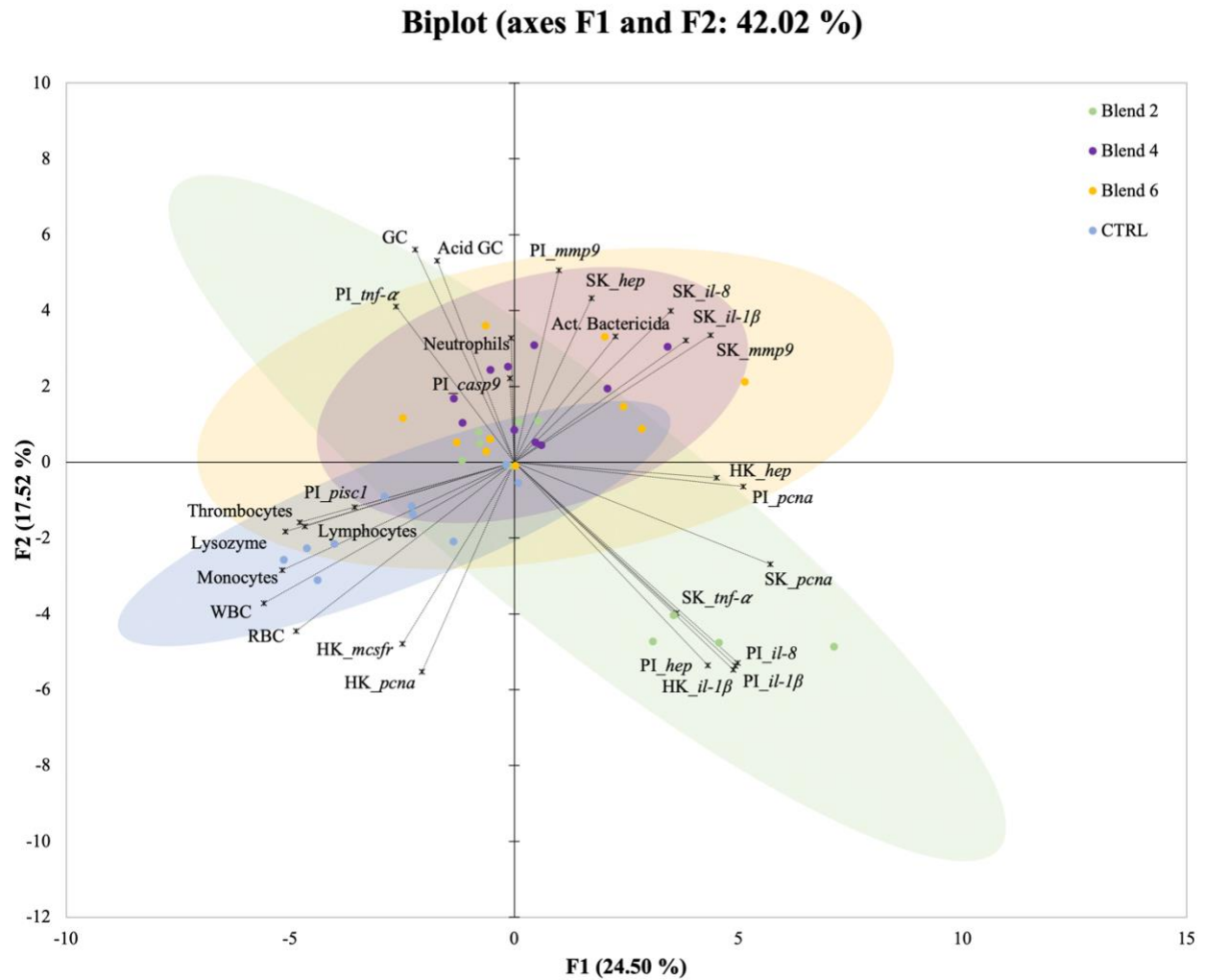


Figure 8. Principal component analysis (PCA) biplot. Scores of each fish are represented as points, and the figure displays the 95% confidence ellipses for each dietary treatment: CTRL – blue, Blend 2 – green, Blend 4 – purple and Blend 6 – yellow. Variables for which significant differences were detected are displayed as loading vectors. HK, head-kidney; SK, skin; PI, posterior intestine; RBC, red blood cells; WBC, white blood cells; GCs, goblet cells; *tnf- α* , Tumor necrosis factor-alpha; *casp9*, Caspase 9; *pcna*, Proliferating cell nuclear antigen; *mmp9*, Matrix-metalloproteinase 9; *mcsfr*, Macrophage colony-stimulating factor receptor 1; *pisc1*, Piscidine 1; *hep*, Hecpidin precursor.

5.4 Discussion

Intensive aquaculture is often equated to a rise in disease outbreaks (Stentiford et al., 2017). Targeted nutritional approaches can ward off diseases in farmed fish. For example, diets containing algae-derived bioactive compounds with immunomodulatory effects (Camacho et al., 2019; Silva et al., 2020) can incapacitate pathogens, thereby contributing to reduction in health risks. *T. maritimum* is the etiological agent of tenacibaculosis, an ulcerative disease that has been causing havoc in European aquaculture by affecting most of the marine farmed fishes including European seabass. In the present study, we have evaluated the mucosal and systemic immune responses in European seabass fed plant-protein rich diets supplemented with a micro- and macroalgae (*N. oceanica*, *C. vulgaris*, *G. gracilis* and *Ulva rigida*) blend (Algaessence™ – Feed) for 12 weeks, and subsequently bath challenged with *T. maritimum*. The results revealed the ability of the algae blend to improve fish growth performance and promote an adequate mucosal immune response upon infection with the pathogen *T. maritimum*.

The CTRL diet with relatively high levels of plant-based products was selected by following the current trends in commercial aquafeeds. However, previous studies have indicated that such diets may impair fish growth (Resende et al., 2022) and/or negatively affect disease resistance (Hua et al., 2019). Hence, it is important to find novel and sustainable products such as algae which have the capacity to negate such challenges faced by the aquaculture industry. The *in vitro* bactericidal activity of the algae blend Algaessence™ – Feed, against *T. maritimum* and other bacterial pathogens, has been previously demonstrated (Ferreira et al., 2021). In the present work, we revealed that the *in vivo* inclusion of this algae blend in plant-protein rich diets for European seabass modulates both local and systemic immune responses and enhances fish growth. Fish haematological profile evidenced a lower number of circulating WBC following infection with *T. maritimum*, particularly lymphocytes and monocytes, in fish fed the blend diets compared to the CTRL fish, which plausibly points to the redistribution of leucocytes from the blood to other body compartments (i.e., the skin and intestine). The mucosal tissues are deeply involved in the defence mechanisms of fish against invading pathogens, with the skin, along with the skin mucus, acting as the primary physical/chemical barrier (Benhamed et al., 2014). Gut mucosal tissue, rich in immune cells, also plays a key role in the maintenance of host homeostasis, by affecting both fish nutritional and immunological status (Lazado & Caipang, 2014). *T. maritimum* is known to initiate the infection process in the mucosal surfaces, specifically through the attachment of the pathogen to the external skin and mucus, followed by infection of other mucosal surfaces (i.e., intestine) and subsequent systemic infection (Avendaño-Herrera et al., 2006).

The hypothesis of leucocyte migration to the mucosal organs is further corroborated by the expression patterns of *mmp9* and *pcna* in the skin and intestine. The gene *mmp9*, encoding matrix-metalloproteinase 9, is involved in both the initial and later phases of inflammation, by promoting leucocyte migration and tissue regeneration. A previous study in zebrafish (*Danio rerio*) has demonstrated a protective role of Mmp9 molecule against *Listeria monocytogenes* infection, by promoting the migration of macrophages to the site of infection (Shan et al., 2016). Likewise, a higher number of PCNA positive cells in tissues of fish countering a pathogen infection may indicate prevention of injury and/or damage repair (Dezfuli et al., 2012; 2017; 2015). Hence, the higher expression of *mmp9* in the skin of fish fed blend 4 compared to CTRL fish, and in the intestine of those fed blends 4 and 6, as well as the up regulation of *pcna* in the mucosal surfaces of all fish that consumed the blend diets suggests an ability of algae-rich diets to promote the migration of the inflammatory cells to the peripheral infected tissues. Besides the lower number of circulating lymphocytes and monocytes observed in fish fed the blend diets compared the CTRL group, an increase in blood neutrophils was also observed in blend 4-fish compared to all other diets, 48 h post-infection. Within the leucocyte's population, neutrophils are the first cells to be recruited to the infected site, initiating an innate immune response (Do Vale et al., 2002). Blood neutrophilia is commonly reported as a reaction to pathogen infection in fish (Costas et al., 2013; Lamas et al., 2005). Guardiola et al. (2019) observed increased number of neutrophils in the blood of Senegalese sole, 48 h after a bath challenge with *T. maritimum*, compared to a non-infected group. The same study reported a correlation between neutrophil counts and plasma peroxidase activity. But in the present study, we did not detect any correlation between plasmatic lytic enzymes and neutrophil counts. Nevertheless, the increased blood neutrophilia in blend 4-fed fish may indicate an added-value of the 4% dietary inclusion of the algae blend to modulate the cell-mediated innate immune response upon *T. maritimum* infection.

This cell migration was accompanied by a proinflammatory response in both the skin and intestine, based on the higher expression of the early response proinflammatory cytokine *il-1 β* and chemokine *il-8*. IL-1 β promotes phagocytosis, lymphocyte proliferation, superoxide production and induces the release of other cytokines (Zhu et al., 2013); IL-8 – produced by monocytes, macrophages, epithelial cells, and the endothelial cells – recruits the leucocytes (mainly the neutrophils) to the inflammatory sites (Köhidaï & Csaba, 1998). Overall, the fish fed the blend diets seem to induce effective immune responses to the presence of the pathogen, i.e., recruitment of inflammatory cells and activation of the pro-inflammatory chemokines and cytokines. Similarly, a recruitment of leucocytes to the inflammatory site, accompanied by an up-regulation of several pro-inflammatory genes, and increased resistance against Phdp infection, was observed in European seabass fed a functional diet supplemented with methionine (Machado et al., 2018).

Besides the cellular immune response in skin and intestine, the 4% incorporation level of the algae blend also led to a higher number of GCs, specifically acid GCs, in the posterior intestine compared to CTRL fish. Such response may indicate an enhancement of the mucosal innate immunity in blend 4-fish, since the acidic mucins produced by acid GCs are often associated with an improved intestinal protection against invading pathogens (Kim & Khan, 2013). Similarly, Passos et al. (2021) reported an increased number of acids GCs in intestine of European seabass fed diets supplemented with *G. gracilis* (2.5% and 5% inclusion), evidencing the intestinal immune boost that algae may confer.

At the systemic level, an increased plasma bactericidal activity was also observed in fish fed all blend diets compared to CTRL fish. Several enzymes in the serum of fish, namely lysozyme, peroxidase, complement, protease and antiprotease, have key roles in the immune response and may contribute to the plasma bactericidal activity (Guardiola et al., 2014). In the present study, the higher plasma bactericidal activity in fish consuming the blend and the increased expression of the antimicrobial peptide hepcidin in the head-kidney (all blends), skin (blend 4) and intestine (blend 4 and 6) compared to CTRL suggest that the algae blend promotes the production of antimicrobial peptides that are known to play a decisive role in the innate immune response as they possess powerful microbicidal activity against a wide range of pathogens (Masso-Silva & Diamond, 2014).

As observed in the PCA biplot, the blend-fed fish were associated with parameters related with a mucosal response as well as plasma bactericidal activity. Instead, CTRL fish were associated with the haematological parameters, plasma lysozyme and immune gene expression in the head-kidney. Indeed, in the CTRL fish, we observed a higher lysozyme activity in plasma coupled with the higher expression of the marker of cell proliferation (*pcna*) and the regulator of mononuclear phagocyte production (*mcsfr1*) in the head-kidney, plausibly indicating a systemic response. Such results may indicate that, after the bath challenge, blend-fed fish were more prepared to promptly fight tenacibaculosis at the local mucosal levels. On the contrary, the skin mucosal surfaces of the CTRL fish were less equipped to fight the pathogen, and the abrasion and damage in the tissues may have resulted in a subsequent systemic infection. A higher number of leucocytes produced in the head-kidney and the circulating lymphocytes and monocytes coupled with the lysozyme activity might, therefore, have promoted the activity at the systemic level to prevent the progression of disease in CTRL-fed fish.

In the present study, European seabass fed the blend diets improved growth performance in comparison to CTRL fish, with blend 6-fish presenting a significantly higher final body weight than all other dietary treatments. However, fish consuming the blend 4 diet presented a clearer immune-modulation in comparison with the other blend-fed groups, accompanied by a lower mortality. This potentially protective effect provided by the blend 4 could be due to different

immune-related responses, namely a higher cellular mucosal immunity, increased plasma bactericidal activity, and a higher number of circulating neutrophils and acidic GCs in intestine. Altogether, the ability of the pathogen to adhere, colonize and degrade the mucosal tissues to eventually cause fish mortality was likely thwarted by blend 4, through the modulation of immune parameters to successfully inhibit the pathogenesis. Such results indicate that the immune modulation promoted by this blend is not strictly associated with fish body weight and suggest that a 4% incorporation level of this micro- and macroalgae blend might be the most adequate to improve the resistance of European seabass juveniles to *T. maritimum* infection. Therefore, the selection of the incorporation level of a functional ingredient is a complex process since its functionality relies on several physiological processes and its protective effects are dependent on the dosage. In a previous study with European seabass consuming diets with *Gracilaria* sp., and subjected to a bacterial challenge with Phdp, a delayed photobacteriosis and decreased mortality (although not significant) was observed in fish fed algae diets (Peixoto et al., 2019). However, the protective effects of algae against tenacibaculosis have not been reported yet.

The present study has provided detailed information regarding the immune responses after a bath challenge with *T. maritimum* following a long-term feeding with algae blend-supplemented diets. From an economic point of view and for the benefit of feeds/fish producers, future studies should reveal the immunomodulatory ability of this algae blend and protective effects against tenacibaculosis (and other fish pathogens) after a short-term feeding period (e.g., 4 weeks); the results will reveal the potential of the algae blend to serve as a functional additive during bacterial infections.

5.5 Conclusion

Here it was evaluated, for the first time, the ability of a commercially produced micro- and macroalgae blend to evoke immunomodulatory activity, both at the systemic and mucosal levels, to thwart *T. maritimum* infection in European seabass. Results indicate that, in a dose-dependent manner, this blend is able to promote a more exacerbated mucosal immune response by favoring the migration of immune cells, particularly lymphocytes and myeloid cells, to the infection sites (i.e., the skin and intestine). These observations were accompanied by a higher expression of genes associated with cell migration and proliferation (*mmp9* and *pcna*) as well as an upregulation of pro-inflammatory genes (*il-1 β* and *il-8*). At the systemic level, the blend seems to promote the production of the antimicrobial peptide hepcidin that may have contributed to the higher bactericidal activity observed in the plasma of the fish fed these diets. The 4% incorporation level, in particular, was associated with a higher number of circulating peripheral neutrophils and acidic GCs in the intestine. Taken together, the

modulation of these immune parameters in fish fed blend 4 might have successfully incapacitated the pathogen, stalled the disease progression, and be responsible for the reduced mortality observed in this group.

The results obtained in this study highlight the importance of the mucosal immune response evoked by the algae blend after a bacterial challenge. Future studies should delve into further optimizing the inclusion levels of this commercial product in functional feeds for not only European seabass juveniles, but also other farmed fish species as well as different development stages (e.g., larvae), to reveal the ability of this algae blend to improve the immune status and promote fish disease resistance.

5.6 Author contributions

MF: manuscript draft, methodology, investigation, and formal analysis. MM: conceptualization, methodology, and writing – review and editing. CSCM: investigation. MRGM: funding acquisition, conceptualization, and writing – review and editing. HA and JS: conceptualization, and writing – review and editing. VK: supervision, and writing – review and editing. BC: conceptualization, methodology, supervision, and writing – review and editing. LV: funding acquisition, conceptualization, methodology, supervision, and writing – review and editing. All authors contributed to the article and approved the submitted version.

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5.8 Conflict of Interest

HA and JS are employed by the companies ALGAplus and Allmicroalgae, respectively, and CSCM PhD grant is partly financed by ALGAplus and Allmicroalgae. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

5.9 Data availability

Data will be made available on request.

5.10 Acknowledgments

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Chapter 6.

An in-depth characterisation of European seabass intestinal segments for assessing the impact of an algae-based functional diet on intestinal health

By

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Abstract

Sustainable farming of fish species depends on emerging new feed ingredients, which can alter the features of the digestive tract and influence animals' overall health. Recent research has shown that functional feeds hold great potential for enhancing fish robustness by evoking appropriate responses at the intestine level. However, there is a lack of extensive and accurate descriptions of the morphology of the gastrointestinal tract of most farmed fish. We have characterised the intestine of European seabass thoroughly, by targeting four segments – anterior, mid, posterior and rectum. Results indicated that the anterior segment is mostly associated with absorption-related features; this segment has the largest absorptive area, the longest villi, and the highest number of neutral goblet cells (GC). The posterior segment and rectum have distinct histomorphometric features, but both seem to be important for immunity, displaying the highest count of acid GC and the highest expression of immune-related genes. The strongest proliferating cell nuclear antigen (PCNA) signal was observed in the anterior intestine and rectum, with PCNA⁺ cells appearing at the base of the villi and the corresponding villi branches. We have also evaluated the impact of a novel feed supplemented with a macro- and microalgae blend and found that there were no differences in terms of growth. However, the alterations observed in the mid intestine of fish fed the blend, such as thickening of the submucosa and lamina propria, an increased number of leucocytes, and higher expression of immune- and oxidative stress-related genes, suggest that algae may have an immunomodulatory effect. In the current article, we have described the morphology and expression patterns of the intestine segments of European seabass in detail and have presented a comprehensive report of the indices and methods used for the semi-quantitative and quantitative histomorphometric assessments, thereby providing useful information for future studies that aim to maintain intestinal health through dietary interventions.

6.1 Introduction

Intestinal health indices are important for monitoring the welfare of farmed fish because some of the emerging new feed ingredients can alter the features of the digestive tract and have an effect on the overall health of animals (Dawood, 2021). The intestine is not only involved in the absorption of nutrients, but it concurrently acts as a physical and chemical barrier to prevent the passage of harmful compounds and microorganisms (Kuz'mina, 2021; Zhang et al., 2020). Nevertheless, extensive and accurate descriptions of the morphology of the gastrointestinal tract of farmed fish are few and far between (Bjørngen et al., 2020; Verdile et al., 2020b).

European seabass (*Dicentrarchus labrax*) is a carnivorous teleost fish of high economic relevance to the Mediterranean region (Vandeputte et al., 2019). However, intensification of its production associated with environmental stress factors and climate change often triggers unwanted diseases that severely affect the economic sustainability of the sector (Ahmed et al., 2019; Fernández Sánchez et al., 2022). The concept of One-Health has prompted the aquaculture industry to search for ingredients capable of promoting fish health and welfare and allowed functional feeds to gain traction (Hayatgheib et al., 2020; Waagbø & Remø, 2020). Macro- and microalgae are valuable sources of bioactive compounds that can act as immune modulators (Jacob-Lopes et al., 2019; Silva et al., 2020). Hence, algae are promising candidates for functional aquafeeds, with potential to boost the immune responses of farmed fish (Gomez-Zavaglia et al., 2019; Nagarajan et al., 2021). To thoroughly understand the functionality of such products, their impact at the intestinal level has to be delineated because this organ is the key site where diet evokes local immune responses (Dawood, 2021).

Integrated approaches, by combining histomorphometric indices and molecular biomarkers, can provide valuable information about the effects of novel feed ingredients on the morphology and functions of the intestine of farmed fish (Ferreira et al., 2023b; Tan et al., 2020; Xie et al., 2021a). It should be noted, however, that morphologic traits can differ among species and along their intestinal tract, and studies on intestinal features still rely on traditional and laborious methods that largely vary among studies. The available literature commonly identifies three main intestinal segments in fish: anterior (or proximal), mid, and posterior (or distal) intestine (Batista et al., 2020a; Bjørngen et al., 2020; Torrecillas et al., 2019; Verdile et al., 2020b). But since the fish intestine does not have easily distinguishable segments like those in mammals, there is currently no consensus on its morphological division and nomenclature (Vatsos, 2021), resulting in difficulties when comparing results across studies. For example, in studies on European seabass, the definition of the anterior and posterior intestines varies. Some define the anterior intestine as the segment from the pyloric caeca to the ileorectal valve, and the posterior intestine as the portion after this valve, i.e. the rectum

(Alix et al., 2017). Others considered the posterior section and rectum as distinct portions of the posterior intestine (Torrecillas et al., 2019). Furthermore, many studies fail to provide an accurate description of the intestinal portions used in their analysis, and the lack of a standardised terminology only adds to the confusion.

Current histological parameters-based determination of the fish intestinal health depends on classical morphological changes, for example diet-induced adverse effects such as villi shortening. Many of the studies that have reported such morphological changes have relied on semi-qualitative analyses (Agboola et al., 2022; Basto-Silva et al., 2022; Iqbal et al., 2022), but quantitative approaches at the intestine level are becoming increasingly popular (Mota et al., 2023; Torrecillas et al., 2019). Moreover, a previous functional analysis based on transcriptomic profiling attributed specific functions to the segments of the European seabass intestine. This analysis reported that the anterior and mid sections are mostly associated with feed digestion and nutrient absorption, whereas the posterior/rectal segments are especially relevant for immune functions (Calduch-Giner et al., 2016). The posterior/rectal regions are also the preferred sections for understanding the diet-induced modulation of the microbial communities (Batista et al., 2016; do Vale Pereira et al., 2019; Ferreira et al., 2022; Gupta et al., 2019). Nonetheless, the lack of a comprehensive study of the morphology and physiology of seabass intestine hampers the ability to selectively modulate specific targeted traits and hence contribute to precision nutrition.

We wanted to establish reference methods and guidelines for morphological studies at the intestine level, and we believe that this information can be used to assess European seabass intestinal health status, so that results can be easily compared with future studies. Hence, we performed an in-depth characterization of four segments of the seabass intestine (i.e., anterior, mid, posterior and rectum) using definable indices and following a holistic approach: 1) fast-track image analysis combined with traditional histology methods; and 2) evaluation of the gene expression patterns in relation to immunity, oxidative stress, and nutrient transport. These methods were then used to understand the impact of a functional diet supplemented with both micro- and macroalgae on the intestinal structure of European seabass. To analyse the histomorphometric traits, both quantitative and semi-quantitative assessments were performed to compare the performance of these two commonly used approaches for evaluating the intestinal health of fish.

6.2 Materials and methods

6.2.1 Ethical statement

Fish rearing and sampling were conducted using routine and animal husbandry practices of commercial farming operations. The fish trial and experimental protocols were approved by

the Ethical Committee of Riasearch Lda. (Murtosa, Portugal) overseen by the National Veterinary Authority (DGAV, Portugal), in compliance with the guidelines of the European Union (Directive 2010/63/EU) and Portuguese law (Decreto-Lei no. 113/2013, de 7 de Agosto) on the protection of animals used for scientific purposes. The study is reported in accordance with the ARRIVE guidelines.

6.2.2 Experimental diets, feeding trial and fish sampling

A commercial algae blend, which is composed of *Gracilaria* sp., *Nannochloropsis* sp. and *Aurantiochytrium* sp., was produced by the Portuguese company ALGAplus (Ílhavo, Portugal). Two isoproteic (51% dry matter, DM) and isoenergetic (23 kJ g⁻¹ DM) diets were formulated and produced by Sparos, Lda. (Olhão, Portugal): a control diet – CTRL –, with moderate levels of fish meal (15%); and an experimental diet with 2% of the algae blend – Blend (Supplementary Figure S1).

The feeding trial was conducted in a saltwater recirculation system at the facilities of Riasearch Unipessoal Lda., Portugal. European seabass (initial body weight 118.6 ± 15.2 g) were randomly assigned to four 350 L fiberglass tanks (i.e., two tanks per dietary treatment) and fed the experimental diets for 8 weeks (54 days). Fish were hand-fed three times a day and at the end of every feeding cycle, apparent satiation of the fish was confirmed through visual observation. The system conditions were as follows: water temperature of 20 °C, salinity of 18‰, flow rate at 700 L/h (200%/h) and 12 h light/12 h dark photoperiod regime.

At the end of the feeding trial, and following a 48-h fasting period, 10 fish per tank were euthanised by anaesthetic overdose (MS222, 150 mg L⁻¹). The different sections of the intestine were sampled, specifically the anterior region (i.e., right after the pyloric ceca), the mid intestine (i.e., the middle portion in between the anterior and posterior sections), the posterior region (i.e., right before the ileorectal valve), and the rectal section (i.e., right after the ileorectal valve). Samples were carefully washed and fixed in 4% formaldehyde (pH 7.0 ± 0.1) for histological evaluation or deep-frozen for gene expression analysis by RT-PCR.

6.2.3 Histomorphological evaluation of the different intestinal segments of European seabass

After fixation, intestine samples were preserved in 70% ethanol. Samples were further processed according to standard histological procedures and embedded in paraffin. Five fish per tank were then selected for histological analyses (10 fish per dietary treatment). Intestinal cross-sections (3 µm) were obtained using a semi-automated rotary microtome (Leica RM 2245) and stained with Alcian Blue/Periodic Acid Schiff (AB/PAS; pH 2.5) and May Grünwald-Giemsa (MGG). Both staining techniques can be used to quantitatively measure almost all the

evaluated parameters, except for the determination of the number of GC (AB/PAS specific) and measurement of microvilli height and leucocyte counting (MGG specific). Micrographs of each individual section were examined under a light microscope (Olympus BX51, Germany) with a camera (Olympus DP50). The sections (i.e., one cross-section per intestinal sample) were analysed using both quantitative (Figure 1 and Supplementary Table S1) and semi-quantitative approaches (Table 1).

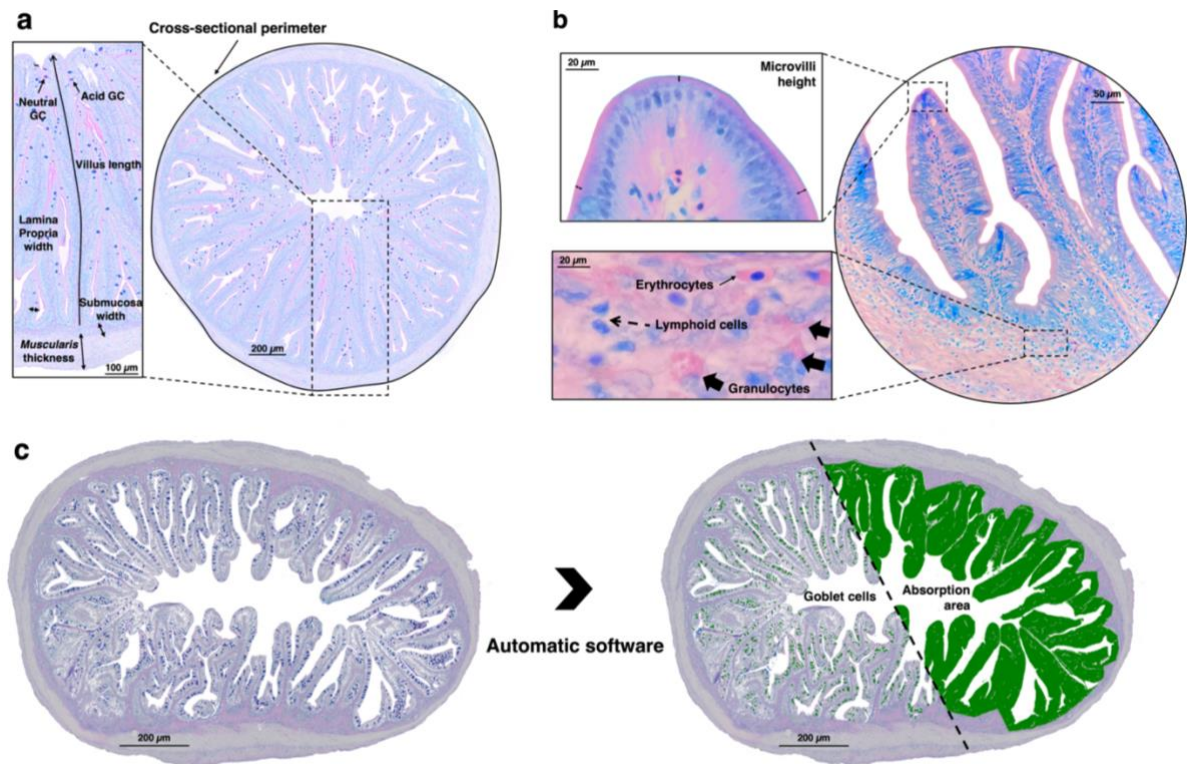


Figure 1. Strategy used for the quantitative analysis of the features of the intestine of European seabass. a) Sections were stained with Alcian Blue/Periodic Acid Schiff (AB/PAS) to detect the acid and neutral goblet cells (GC), and measure the villi length, the submucosa and lamina propria width, the muscularis thickness and the cross-sectional perimeter. b) Sections stained with May Grünwald-Giemsa (MGG) were used to measure the microvilli height, and to count the lymphoid cells and granulocytes in the submucosa and lamina propria. Note the morphology of the different cells found in submucosa and lamina propria: granulocytes are big, oval-shaped cells, with pink granules in the cytoplasm and a blue-stained nucleus in the periphery; lymphoid cells are small cells that stain blue; and erythrocytes are elongated cells with a pink cytoplasm and centrally located blue-stained nucleus. c) The number of acid and neutral GCs and the absorption area (total area occupied by the villi) were automatically determined by the software. All measurements were performed using the imaging software Olympus cellSens. See Table 2 for more details about the quantitative analysis.

Table 1. Description of the scores used to evaluate the intestinal morphology of European seabass – semi-quantitative analysis.

Parameter	Score	Description
Villi length and integrity ^a	1	Mucosal layer with a regular columnar epithelium with polarized and basally located nuclei, with no tissue damage. Anterior and rectum sections: long and thin organized villi. Mid and posterior sections: medium villi.
	2	Typical villi length described in 1 for each section, and very few irregular/stubby folds (less than 1/4 of the section).
	3	Typical villi length, and few irregular/stubby folds (1/4 of the section).
	4	Reduction on the typical villi length, and few irregular/stubby folds (1/4 of the section).
	5	Reduction on the typical villi length, and some irregular/stubby folds (1/2 of the section).
	6	Short villi, and many irregular/stubby folds (1/2 to 3/4 of the section).
	7	Short villi, and most folds were irregular/stubby (3/4 of the section).
	8	Very short villi, and most folds were irregular/stubby (3/4 of the section) with tissue damage.
	9	Very short and irregular villi, with apical displacement of nuclei and damaged tissue.
Submucosa width ^b	1	Mid, posterior and rectum sections: thin connective tissue between the base of the villi and the muscular layer. Anterior section: very thin submucosa layer.
	2	Slightly wider connective tissue beneath very few villi (less than 1/4 of the section).
	3	Wider connective tissue beneath few villi (1/4 of the section).
	4	Wider connective tissue beneath some villi (1/2 of the section).
	5	Markedly wider connective tissue beneath some villi (1/2 of the section).
	6	Markedly wider tissue beneath many villi (1/2 to 3/4 of the section).
	7	Thick layer of the connective tissue beneath many villi (1/2 to 3/4 of the section).
	8	Thick layer of the connective tissue beneath most villi (more than 3/4 of the section).
	9	Extremely thick layer of connective tissue beneath all villi.
Lamina propria width ^b	1	Anterior, mid, and posterior sections: thin lamina propria and delicate core of connective tissue. Rectum: lamina propria is very thin and almost imperceptible.
	2	Slightly wider lamina propria in very few villi (less than 1/4 of the section).
	3	Wider lamina propria in few villi (1/4 of the section).
	4	Lamina propria appears wider in some villi (1/2 of the section).
	5	Lamina propria was markedly wider in some villi (1/2 of the section).
	6	Lamina propria was markedly wider in many villi (1/2 to 3/4 of the section).
	7	Lamina propria is thick in many villi (1/2 to 3/4 of the section).
	8	Lamina propria is thick in most villi (more than 3/4 of the section).
	9	Lamina propria is very thick in all villi.
Submucosa lymphoid cells	1	Anterior, mid, and posterior sections: lymphoid cells sparsely present in the submucosa. Rectum: monolayer of lymphoid cells appearing right beneath the villi, and lymphoid cells sparsely spread out through the rest of the submucosa.
	2	Slight increase in the number of lymphoid cells in the submucosa compared to the normal appearance.
	3	Anterior, mid, and posterior sections: lymphoid cells spread at a higher density (per area) throughout the submucosa. Rectum: few layers of lymphoid cells present right beneath the villi, and lymphoid cells sparsely spread out through the rest of the submucosa.
	4	Marked increase in the number of lymphoid cells in the submucosa.
	5	Submucosa thoroughly filled with lymphoid cells.
Submucosa granulocytes	1	Granulocytes sparsely present in the submucosa.
	2	Slight increase in the number of granulocytes in the submucosa.
	3	Granulocytes spread at a higher density throughout the submucosa.
	4	Marked increase in the number of granulocytes in the submucosa.
	5	Submucosa heavily filled with granulocytes.
Lamina propria lymphoid cells	1	Anterior, mid, and posterior sections: lymphoid cells sparsely present in the lamina propria and sometimes present right beneath the enterocytes. Rectum: almost no lymphoid cells visible in lamina propria.
	2	Slight increase in the number of lymphoid cells in the lamina propria compared to the normal appearance.
	3	Lymphoid cells spread throughout lamina propria at a higher density and intraepithelial lymphoid cells located at the base of the enterocytes.
	4	Marked increase in the number of lymphoid cells in the lamina propria and intraepithelial lymphoid cells located at the base and/or apex of the enterocytes.
	5	Lamina propria thoroughly filled with lymphoid cells and high density of intraepithelial lymphoid cells, located at the apex of the enterocytes.
Lamina propria granulocytes	1	Anterior, mid, and posterior sections: granulocytes sparsely present in the lamina propria. Rectum: almost no granulocytes are visible in lamina propria.
	2	Anterior, mid, and posterior sections: Slight increase in the number of granulocytes in the lamina propria compared to the normal appearance. Rectum: few granulocytes spread throughout the lamina propria.
	3	Anterior, mid, and posterior sections: granulocytes spread throughout the lamina propria at a higher density (per area). Rectum: some granulocytes spread throughout all lamina propria.
	4	Marked increase in the number of granulocytes in the lamina propria.
	5	Lamina propria filled with granulocytes.

^aAdapted from Urán et al. (2008), Silva et al. (2015) and Penn et al. (2011); ^bAdapted from Silva et al. (2015) and Penn et al. (2011)

A detailed description of the different histomorphological parameters evaluated quantitatively in the intestine of European seabass can be found in Supplementary Table S1. Briefly, two sections per sample were examined using an imaging software (Olympus cellSens Standard 2.2). The AB/PAS-stained section was used to determine the cross-sectional perimeter (CSP), the absorption area, the villi length, the muscularis thickness, the submucosa thickness, the lamina propria width and the number of goblet cells (GC), namely the acid and neutral GC (Figure 1a). The ratios villi length/CSP, GC/absorption area, acid GC/absorption area, and neutral GC/absorption area were also calculated. On the other hand, the MGG-stained section was used to measure the microvilli height, and to manually count the number of lymphoid cells and granulocytes in the submucosa and lamina propria (Figure 1b). As seen in Figure 1c, the total number of acid and neutral GC and the absorptive area (total area occupied by the villi) were automatically determined using the analysis tools of the Olympus cellSens software.

An immunohistochemical procedure was used to determine the localization of the proliferating cells using a cell nuclear antigen (PCNA). Firstly, the sections were dewaxed and rehydrated. Then antigen was retrieved by immersing the slides in 0.01 M citrate buffer (pH 6.0), followed by irradiation on a microwave on high power (800 W) for 20 min. After cooling at room temperature, the slides were rinsed for 5 min in running tap water, and immunostained using the Novolink Polymer Detection System kit (RE7140-K, Leica Biosystems, Germany), according to the manufacturer's instructions. Thereafter the sections were incubated overnight with the primary antibody – anti-PCNA mouse monoclonal antibody (Santa Cruz Biotechnology, EUA) in a humidified chamber, at 4 °C. The antibody was diluted 1:2000 in 1% bovine serum albumin (BSA)/phosphate buffered saline (PBS). Counterstaining was performed with haematoxylin. The area occupied by PCNA⁺ cells was automatically determined by the Olympus cellSens software, using the same procedures that were adopted for counting the GC (Figure 1c and Supplementary Table S1).

Each MGG stained section was also semi-quantitatively evaluated according to the criteria outlined in Table 1. To evaluate each intestinal segment's features, all samples were screened beforehand to ensure the accurate assignment of the score of 1, as morphologic traits vary throughout the tract. For example, in the anterior and rectum sections, a score of 1 indicates long, thin, well-organized villi with no tissue damage, while in the mid and posterior sections, it corresponds to medium-length villi with no tissue damage. Furthermore, as scores increase, so does the degree of alteration in comparison to score 1. This example demonstrates the importance of carefully assessing each section's specific morphologic traits to assign accurate and reproducible scores. Concerning the scores range, while villi length and integrity, submucosa thickness, and lamina propria width were assigned scores of 1-9, submucosa lymphoid cells, submucosa granulocytes, lamina propria lymphoid cells, and lamina propria

granulocytes were assigned scores of 1-5. These scores were considered the most appropriate to define the different intrinsic intestinal segment's features.

6.2.4 Expression of genes in the different intestinal sections of European seabass

Total RNA was extracted from the different intestinal sections (~30 mg; n = 16 per treatment), using TRIzol reagent (Invitrogen, USA) and NZY Total RNA Isolation kit (NZYTech, Portugal), according to the method described by Ferreira et al. (2020). The quantity and purity of the extracted RNA were spectrophotometrically evaluated, while RNA integrity was assessed using an agarose electrophoresis gel as previously described (Ferreira et al., 2023b). Using 1 µg of RNA, first strand cDNA was synthesized, and real-time PCR (RT-PCR) assays were performed on a CFX384 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, USA) with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, USA). Following the protocol described by Ferreira et al. (2023b), reactions were performed in duplicate. The employed thermal cycling conditions were: 95 °C for 30 sec, followed by 35 cycles of two steps of 95 °C for 5 sec and 60 °C for 30 sec. The specificity of the RT-PCR reaction was ensured by a post-amplification dissociation curve, while PCR efficiency for each gene was determined using a 5-fold serial dilution of cDNA from all the samples used in the experiment. A total of 22 genes (four reference genes and 18 target genes) were analysed, including genes associated with a) immunity (i.e., *igM*, immunoglobulin M; *cd4*, cluster of differentiation 4; *il-6*, interleukin-6; *il-8*, interleukin-8; *il-1β*, interleukin-1 beta; *tnf-α*, tumor necrosis factor-alpha; *tcrβ*, T-cell receptor antigen receptor beta chain; *tlr9*, toll-like receptor 9; *casp3*, caspase 3; *pcna*, proliferating cell nuclear antigen; *pisc1*, piscidine 1); b) oxidative stress (i.e., *gpx*, glutathione peroxidase; *sod*, superoxide dismutase; *cat*, catalase); and c) nutrient digestion and absorption (i.e., *alp*, alkaline phosphatase; *malt*, maltase; *fabp2*, intestinal fatty acid binding protein; *aqp1*, aquaporin 1). The primer sequences, accession numbers, annealing temperatures, and PCR efficiencies can be found in Supplementary Table S2. The stability of the reference genes (i.e., *18s*, *β-actin*, *ef1α* and *gapdh*) was determined by the geNorm algorithm (Vandesompele et al., 2002), within the qbase + software, version 3.2 (Biogazelle, Zwijnaarde, Belgium - www.qbaseplus.com), and a normalization factor was calculated for each sample. The comparative critical threshold ($\Delta\Delta CT$) method (Livak & Schmittgen, 2001) was used to calculate the relative abundance of the target genes, as described by Ferreira et al. (2023b).

6.2.5 Statistical analysis

The IBM SPSS software platform 27.0 (SPSS Inc., Chicago, IL, USA) was used to perform statistical analysis. The assumptions of normality and homogeneity of variances were checked by Kolmogorov-Smirnov and Levene's tests, respectively. Data were transformed when necessary. For comparisons between intestinal sections, one-way ANOVA followed by Tukey's multiple comparison test was performed. The level of significance used was $P < 0.05$. Independent samples t-test was applied to understand the differences between the two diets. A Spearman's rank correlation coefficient test was applied to the histology-based variables of the quantitative and semi-quantitative data. Significant correlations were considered at the bilateral levels of 0.05 or 0.1. The principal component analysis (PCA) was performed using XLSTAT version 2022.1.2. (Addinsoft, USA) to differentiate the samples from the different study groups.

6.3 Results

6.3.1 Histomorphology of the different sections of the intestine of European seabass

Quantitative analyses were performed to describe the micromorphology of the different intestinal sections. The results of the analyses are presented in Table 2 and the differences in the morphology of anterior, mid, posterior, and rectal sections are evidenced in Figure 2.

The anterior region had the largest absorptive area, the longest villi, the thinnest submucosa, and the largest total area of PCNA⁺ cells compared to all other sections. The posterior and rectal sections had higher GC and acid GC counts than the anterior and mid regions. The average area of the GC (including acid and neutral cells) was also significantly larger in these sections (i.e., posterior intestine and rectum). As for the GC and acid GC counts expressed per intestinal absorptive area, the highest values were for the posterior intestine, followed by the mid intestine and rectum, with the anterior intestine presenting the lowest value in comparison with all other sections. On the other hand, the anterior section had the highest count of neutral GC compared to the mid and posterior regions. However, when such neutral GC counts were expressed per absorptive area, we did not detect any significant differences between sections. The anterior and mid regions had significantly higher number of granulocytes in submucosa and lamina propria, and lymphoid cells in lamina propria compared to the posterior and rectal sections. The rectum had the thickest muscularis, the narrowest lamina propria, the longest microvilli, and the highest lymphoid cells counts in submucosa, in comparison with the other intestinal regions.

Concerning the PCNA⁺ cells per absorptive area, the anterior intestine presented the highest values, but was not significantly different from the rectum. Moreover, as observed in Figure 2,

the PCNA signal in the anterior portion was detected in the basal part of the complex folds and in the villi branching points, while in rectum the PCNA⁺ cells were concentrated at the basis of the villi.

A semi-quantitative analysis with well-defined and validated scoring system is described in Table 1 and was applied to the different intestinal sections in order to describe the intrinsic intestinal segment's features (Supplementary Table S3). Likewise, the quantitative analyses, the semi-quantitative approach confirmed that the anterior intestine is characterized by long villi with very few irregular folds and a thin submucosa layer. The mid and posterior segments had medium sized villi with few irregular folds, and the rectum had a very thin lamina propria with very few granulocytes. It is important to note that the attributed scores are specific to each section and cannot be used to compare differences between them.

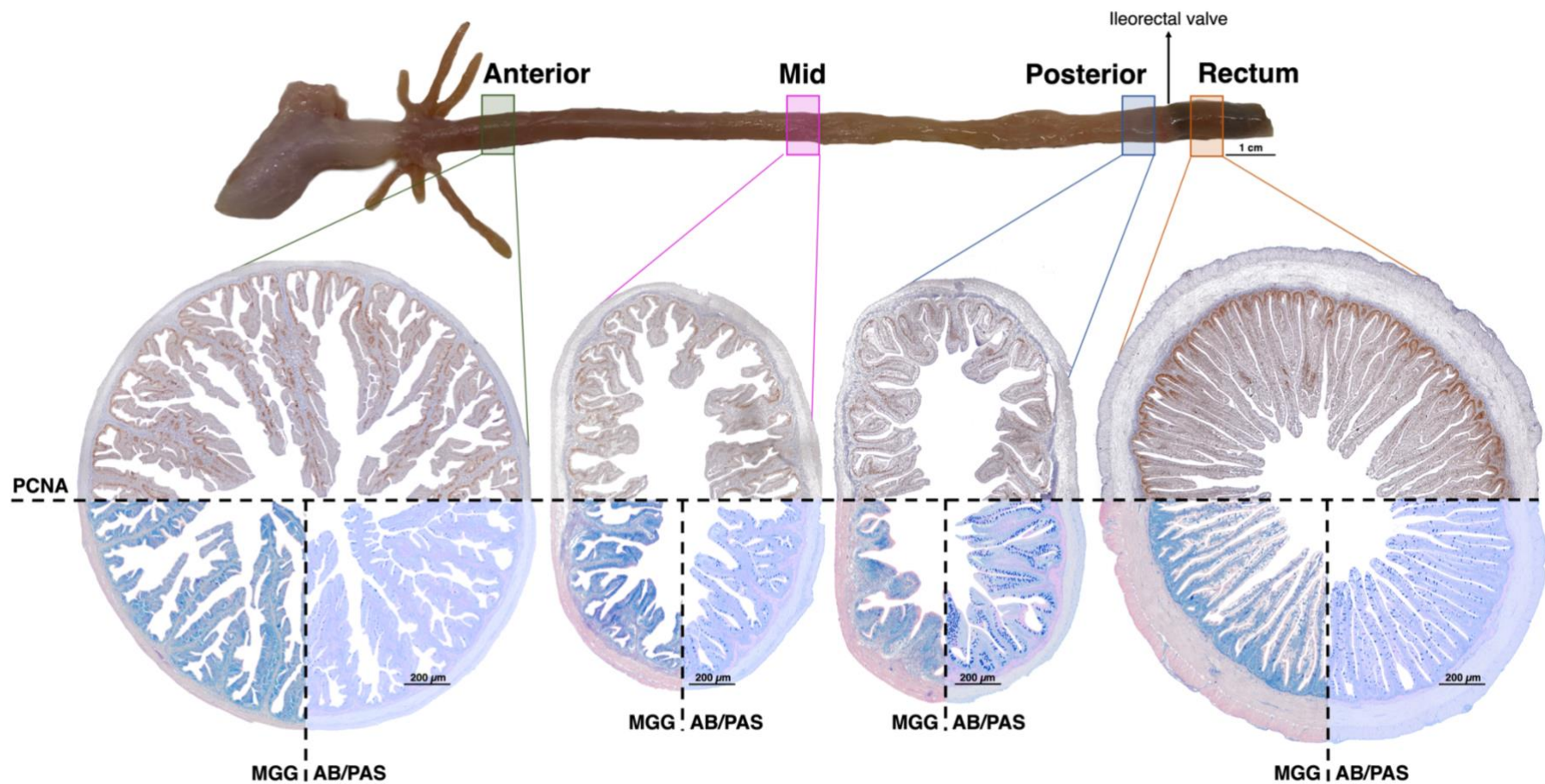


Figure 2. Histomorphology of the different sections of the intestine of European seabass. PCNA, Proliferating cell nuclear antigen (immunohistochemistry); MGG, May Grünwald-Giemsa (staining); AB/PAS, Alcian Blue/ Periodic Acid Schiff (staining). Note the morphological differences as well as the location of the PCNA⁺ cells that characterize the distinct sections.

Table 2. Histomorphology of the different intestine sections of European seabass using a quantitative analysis

	Intestine Sections			
	Anterior	Mid	Posterior	Rectum
Cross-sectional perimeter (CSP, mm)	14.4 ± 1.1 ^a	11.0 ± 1.8 ^b	10.3 ± 1.5 ^b	13.6 ± 1.9 ^a
Absorption area (mm ²)	8.0 ± 1.1 ^a	3.4 ± 0.9 ^b	2.6 ± 0.9 ^c	4.3 ± 1.1 ^b
Muscularis thickness (µm)	112.8 ± 26.7 ^b	135.2 ± 21.0 ^b	128.3 ± 27.2 ^b	263.8 ± 51.2 ^a
Submucosa width (µm)	48.7 ± 12.1 ^b	77.7 ± 20.9 ^a	72.6 ± 23.6 ^a	72.0 ± 11.8 ^a
Lamina propria width (µm)	70.6 ± 10.7 ^a	70.5 ± 16.1 ^a	65.4 ± 12.0 ^a	18.8 ± 6.1 ^b
Villi length (µm)	1456.6 ± 160.3 ^a	780.9 ± 118.0 ^{bc}	577.8 ± 65.5 ^c	900.7 ± 114.7 ^b
Villi length/CSP (µm/mm)	101.5 ± 9.6 ^a	72.3 ± 13.5 ^b	56.6 ± 6.9 ^c	67.2 ± 10.3 ^b
Microvilli height (µm)	4.2 ± 0.6 ^b	3.3 ± 0.4 ^c	4.3 ± 1.0 ^b	5.6 ± 0.7 ^a
Goblet cells (GC)	2189.8 ± 601.5 ^b	2415.7 ± 727.9 ^b	3384.7 ± 974.4 ^a	3700.9 ± 1325.6 ^a
Acid GC	1883.3 ± 578.5 ^b	2295.1 ± 775.0 ^b	3273.3 ± 1036.5 ^a	3451.3 ± 1252.0 ^a
Neutral GC	306.5 ± 198.6 ^a	120.6 ± 154.8 ^b	111.4 ± 151.1 ^b	249.6 ± 287.1 ^{ab}
GC/absorption area (no. GC/mm ²)	291.8 ± 88.2 ^c	718.5 ± 153.5 ^b	1287.8 ± 233.4 ^a	869.1 ± 210.0 ^b
Acid GC/absorption area (no. GCA/mm ²)	249.3 ± 88.6 ^c	682.2 ± 173.9 ^b	1236.4 ± 236.8 ^a	809.6 ± 193.0 ^b
Neutral GC/absorption area (no. GCN/mm ²)	42.6 ± 21.2	36.3 ± 47.2	51.4 ± 74.6	59.5 ± 63.3
Average area GC (µm ²)	33.3 ± 7.2 ^b	34.9 ± 6.3 ^b	51.7 ± 10.9 ^a	58.0 ± 10.7 ^a
Submucosa lymphoid cells	65.6 ± 25.6 ^b	85.9 ± 34.8 ^b	66.9 ± 17.8 ^b	187.8 ± 66.6 ^a
Submucosa granulocytes	28.2 ± 12.2 ^a	30.3 ± 9.9 ^a	17.9 ± 6.0 ^b	16.3 ± 4.8 ^b
Lamina propria lymphoid cells	57.8 ± 24.6 ^a	57.2 ± 23.2 ^a	34.3 ± 9.6 ^b	23.3 ± 8.4 ^b
Lamina propria granulocytes	15.6 ± 5.8 ^a	13.1 ± 4.8 ^a	5.4 ± 1.5 ^b	3.1 ± 1.3 ^b
PCNA ⁺ cells total area (mm ²)	0.8 ± 0.2 ^a	0.2 ± 0.1 ^c	0.1 ± 0.1 ^c	0.3 ± 0.1 ^b
PCNA ⁺ cells total area/absorption area (%)	7.4 ± 2.5 ^a	5.3 ± 3.0 ^c	5.6 ± 2.3 ^{bc}	6.4 ± 2.5 ^{ab}

Values presented as mean ± SD (n = 20 samples per section). One-way ANOVA followed by Tukey's multiple comparison test was performed. Different letters denote significant differences between intestinal sections. PCNA, Proliferating cell nuclear antigen. Figure 2 provides a visual representation of the characteristics of the distinct sections.

6.3.2 Gene expression patterns of the different sections of the intestine of European seabass

A panel of 18 genes, involved in immunity, oxidative stress and nutrient digestion and absorption, were selected to determine the gene expression patterns associated with the different sections of the intestine of European seabass (Figure 3). The expression of genes involved in the different immune pathways was, in general, higher in the rectum. Specifically, the mRNA levels of *il-8*, *il-1β* and *tlr9* were significantly higher in the rectum compared to all other sections. The genes *cd4*, *tnf-α*, and *tcrlβ* had higher expression in rectum only in comparison with the anterior and mid regions. In the rectum, the expression of *igM* and *casp3* was higher compared to only the mid intestine. The expression of *il-6* was higher in both the

anterior and rectal section but compared to only the mid segment. In contrast, the *pcna* gene was found to have the highest transcript levels in the anterior region, followed by the rectum, with the mid region presenting the lowest expression values. There were no significant differences between the expression of the *pisc1* gene in the different intestinal sections (Figure 3a).

The relative expression of genes associated with oxidative stress (i.e., *sod* and *cat*) differed significantly between the intestinal sections. The genes *sod* and *cat* were significantly downregulated in the rectum compared to all the other regions. The intestinal sections had similar levels of the mRNA of the gene *gpx* (Figure 3b).

Concerning the genes related to nutrient digestion and absorption, the highest expression of the *malt* gene was observed in the anterior region, followed by the mid and posterior intestine, with the rectum presenting the lowest expression of this gene. While the *fabp2* gene was significantly lower in the rectum compared to all the other sections, the expression of *aqp1* was lower in the anterior region compared to the mid, posterior, and rectal sections. We did not observe any significant differences between the expression pattern of the *alp* gene along the intestinal tract (Figure 3c).

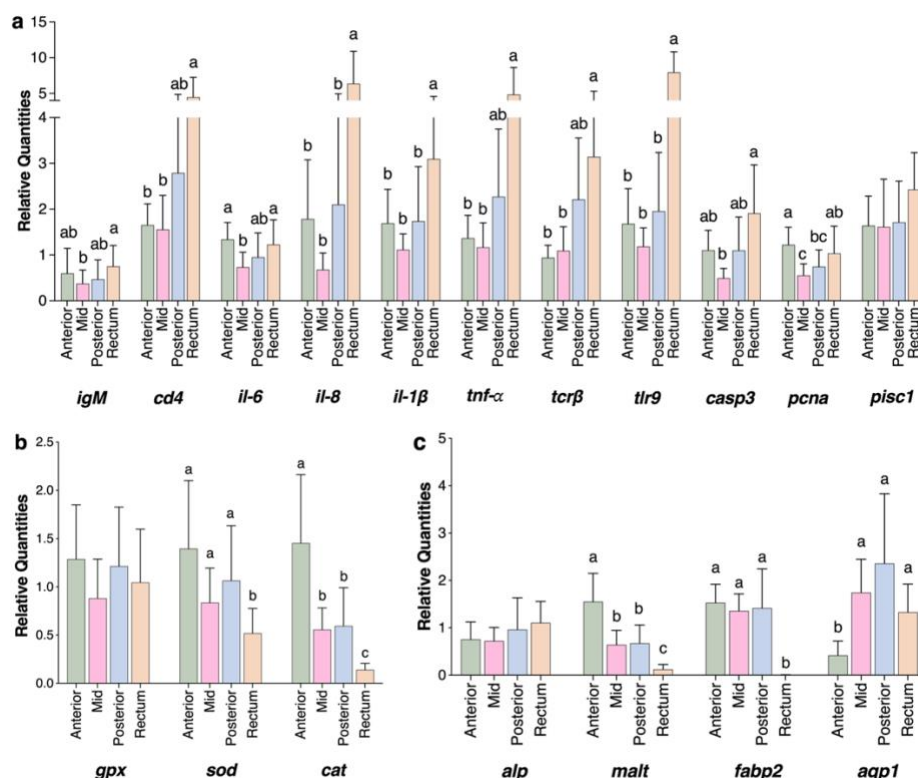


Figure 3. Gene expression in the different sections of the intestine of European seabass juveniles. a) Immune-related genes; b) genes associated with oxidative stress genes; and c) genes related to nutrient digestion and absorption. Relative quantities in the intestine are presented as mean $\pm$ SD (n = 16 samples per section). One-way ANOVA followed by Tukey's multiple comparison test was

performed, and different letters above the bars denote significant differences between intestinal sections.

6.3.3 Principal component analysis – morphology and functionality of the different intestinal sections of European seabass

Principal component analysis (PCA) was employed to visualise all the variables analysed in the anterior, mid, posterior, and rectal portions of the intestine of European seabass. The different intestinal sections were separated sequentially (as in the intestine) along the F1 axis, with the first two dimensions of the PCA biplot explaining 46.34% of the variability in the experimental data (Figure 4). The variables, absorptive area, villi length, expression of the genes *malt*, *fabp2*, *sod* and *cat*, cellular proliferation parameter (PCNA⁺ cells), granulocytes in submucosa and lamina propria, and lymphoid cells in lamina propria were found to be associated with the anterior intestinal section. The immune-related genes, muscularis thickness, microvilli height, acid GC, submucosa width, and expression of the genes *aqp1* and *alp* appeared in the quadrants where the posterior and rectum samples clustered in the PCA plot. The mid samples were in a different quadrant than the other sections (F1 positive/F2 negative).

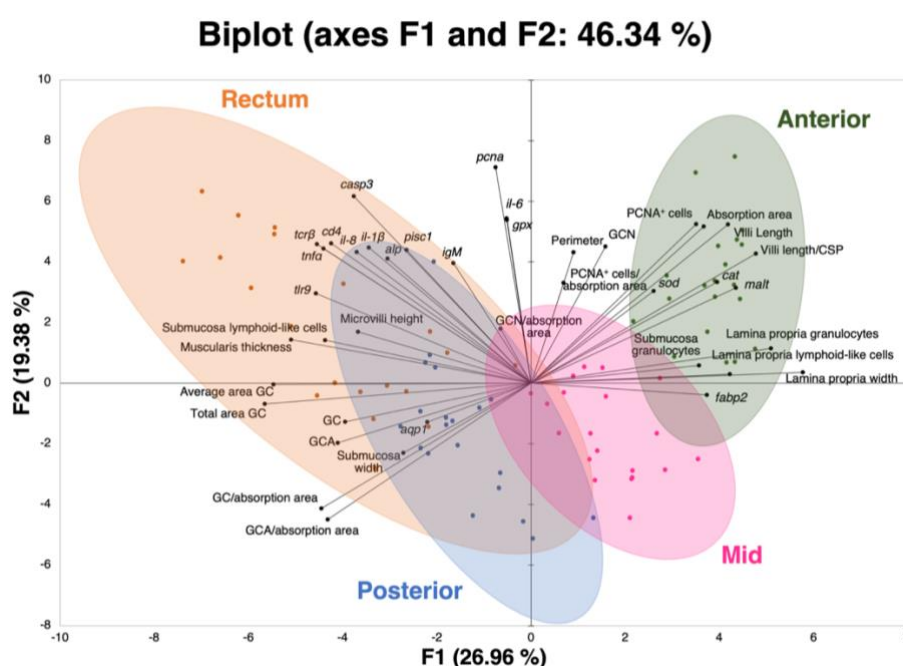


Figure 4. Principal component analysis (PCA) biplot for all variables analysed. Variables are displayed as loading vectors. Confidence ellipses (95%) are shown for each intestinal section: Anterior intestine – dark green; Mid intestine – pink; Posterior intestine – blue; and Rectum – orange.

6.3.4 Growth performance of European seabass fed the experimental diets

After an 8-week feeding period, there was no significant difference in final body weight, weight gain, specific growth rate (SGR), and feed conversion ratio (FCR) of European sea bass that were fed with a diet containing 2% of a commercial algae blend composed of *Gracilaria* sp., *Nannochloropsis* sp. and *Aurantiochytrium* sp. compared to those fed with a CTRL diet (Figure 5).

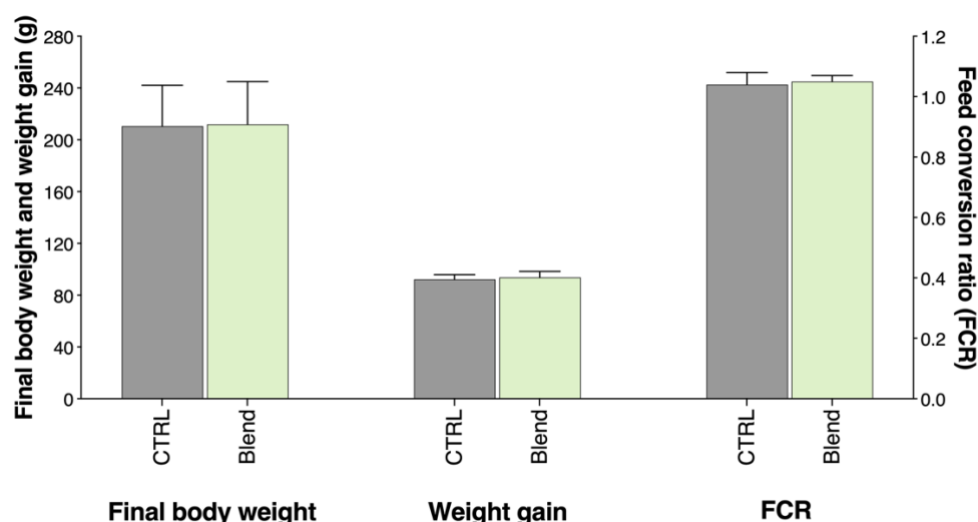


Figure 5. Growth performance of European seabass fed the control or the blend diet. Results are displayed as mean $\pm$ SD (n = 34 fish per treatment). FCR, feed conversion ratio.

6.3.5 Impact of the algae blend-supplemented diet on the intestine of European seabass

To understand the impact of the algae blend-supplemented diet on the intestine of European seabass, both the histomorphology and gene expression patterns were analysed in the fish fed the CTRL and the Blend diets. Overall, the algae blend had very limited impacts on the morphology and functionality of the intestine, with most of the observed differences being located in the mid intestine (Table 3 and Supplementary Figure S2). A reduction in the villi length, number of GC and acid GC, and the total area occupied by the GC was observed in the mid intestine of fish fed the Blend diet compared to those fed the CTRL diet. The mid intestine of the fish fed the blend had thicker submucosa and wider lamina propria, both parts with a significantly higher number of lymphoid cells, and submucosa with more granulocytes. The area occupied by the PCNA⁺ cells per absorptive area in the mid intestine was also significantly increased after the blend diet feeding (Table 3). Alterations of the gene expression patterns were restricted to the mid region, with an upregulation of the *il-8*, *pisc1* and *gpx* genes in fish that consumed the algae-supplemented diet (Supplementary Figure S2). The

differences observed in the other intestinal sections, in response to the blend diet, were limited to a higher number of lymphoid cells counts in the submucosa of the rectum, and higher number of granulocytes in the submucosa of the anterior and rectal sections (Table 3).

A PCA biplot created using all the analysed variables in each intestinal section could not discriminate between the CTRL and Blend samples (Supplementary Figure S3). On the other hand, when only the variables significantly affected by the experimental diet in the mid intestine were selected and visualised in a PCA biplot, the CTRL and Blend samples were separated along the F1 axis, with the first two dimensions explaining 60.64% of the variability of the data (Figure 6). The blend samples were associated with immune-related parameters such as expression of *il-8* and the antimicrobial peptide *pisc1*, the presence of the lymphoid cells in submucosa and lamina propria, and the presence of granulocytes in submucosa. The lamina propria width and submucosa thickness and the oxidative stress-related gene *gpx* were also found in the F1 positive quadrant, where the blend samples were mostly clustered. The variables villi length, GC, acid GC, acid GC per absorptive area and total area occupied by the GC were positioned on the F1 negative quadrant and seemed to be associated with the control samples.

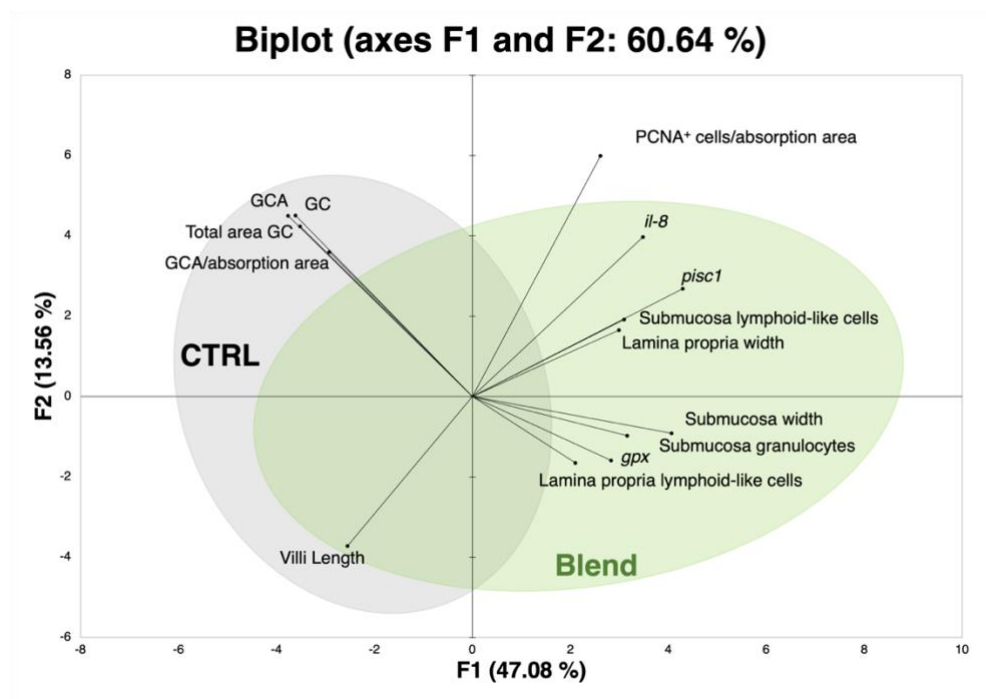


Figure 6. Principal component analysis (PCA) biplot for the variables that displayed significant differences in the mid intestine. Variables are displayed as loading vectors. Confidence ellipses (95%) are shown for each dietary treatment: CTRL – grey; Blend – green.

1 **Table 3.** Histomorphology of the different intestine sections of European seabass fed the control or the blend diets using a quantitative analysis.

	Anterior Intestine		Mid Intestine		Posterior Intestine		Rectum	
	CTRL	Blend	CTRL	Blend	CTRL	Blend	CTRL	Blend
Cross-sectional perimeter (CSP, mm)	14.3 ± 1.2	14.4 ± 1.0	11.4 ± 1.9	10.6 ± 1.5	10.6 ± 1.4	10.1 ± 1.7	13.7 ± 2.0	13.5 ± 2.0
Absorption area (mm ²)	7.8 ± 1.1	8.2 ± 1.2	3.7 ± 1.0	3.1 ± 0.6	2.6 ± 0.6	2.7 ± 1.2	4.4 ± 1.1	4.09 ± 1.1
Villi length (µm)	1498.9 ± 184.3	1414.3 ± 127.8	837.1 ± 119.3 ^a	724.6 ± 90.6 ^b	580.6 ± 68.3	574.9 ± 66.1	939.8 ± 109.6	851.9 ± 107.7
Villi length/CSP (µm/mm)	104.9 ± 9.4	98.2 ± 9.0	74.8 ± 14.4	69.92 ± 12.9	55.1 ± 6.2	58.0 ± 7.6	69.7 ± 10.3	64.0 ± 9.9
Microvilli height (µm)	4.1 ± 0.5	4.2 ± 0.7	3.3 ± 0.5	3.3 ± 0.4	4.2 ± 0.9	4.5 ± 0.6	5.2 ± 0.8	5.9 ± 0.6
Muscularis thickness (µm)	101.8 ± 22.8	123.8 ± 26.8	128.1 ± 20.0	142.3 ± 20.6	117.5 ± 13.9	139.0 ± 33.4	253.9 ± 37.9	276.2 ± 65.0
Submucosa width (µm)	45.8 ± 5.0	51.6 ± 16.3	62.2 ± 12.4 ^b	93.1 ± 15.5 ^a	64.2 ± 9.3	81.0 ± 30.56	67.90 ± 12.54	77.14 ± 8.96
Lamina propria width (µm)	64.6 ± 9.0 ^b	76.6 ± 9.0 ^a	61.1 ± 8.2 ^b	78.5 ± 17.3 ^a	63.6 ± 14.6	67.3 ± 9.2	18.2 ± 5.4	19.5 ± 7.3
Goblet cells (GC)	2206.9 ± 652.8	2172.6 ± 580.6	2871.4 ± 705.7 ^a	2010.6 ± 481.1 ^b	3446.8 ± 1032.3	3322.7 ± 971.3	3656.8 ± 1395.5	3756.0 ± 1325.7
Acid GC	1901.9 ± 596.7	1864.6 ± 591.4	2810.9 ± 696.4 ^a	1836.7 ± 523.9 ^b	3347.0 ± 1040.3	3199.6 ± 1090.3	3475.4 ± 1386.4	3421.3 ± 1154.7
Neutral GC	305.0 ± 233.0	308.0 ± 170.3	60.5 ± 62.2	174.0 ± 194.2	99.8 ± 149.6	123.1 ± 160.6	181.4 ± 208.7	334.8 ± 359.4
GC/absorption area (no. GC/mm ²)	306.7 ± 80.9	278.6 ± 97.0	789.5 ± 154.6	655.3 ± 129.1	1310.8 ± 221.4	1264.8 ± 256.1	827.9 ± 199.1	920.6 ± 225.1
Acid GC/absorption area (no. AGC/mm ²)	260.8 ± 83.8	239.0 ± 96.5	775.1 ± 162.9 ^a	599.6 ± 144.8 ^b	1268.0 ± 199.1	1204.8 ± 278.0	784.2 ± 193.7	841.4 ± 200.5
Neutral GC/absorption area (no. NGC/mm ²)	45.9 ± 24.1	39.6 ± 19.4	14.5 ± 10.4	55.7 ± 58.9	42.9 ± 71.1	60.0 ± 81.3	43.7 ± 46.8	79.3 ± 78.3
Average area GC (µm ²)	30.7 ± 3.7	36.0 ± 9.0	36.3 ± 4.0	33.7 ± 7.8	54.5 ± 13.6	48.8 ± 6.9	58.1 ± 8.6	58.0 ± 13.5
Total area GC (mm ²)	0.07 ± 0.02	0.08 ± 0.02	0.10 ± 0.02 ^a	0.07 ± 0.02 ^b	0.19 ± 0.08	0.16 ± 0.04	0.21 ± 0.07	0.20 ± 0.05
Submucosa lymphoid cells	64.5 ± 8.6	66.7 ± 8.0	69.4 ± 9.5 ^b	102.4 ± 10.3 ^a	67.7 ± 6.6	66.1 ± 4.8	177.1 ± 19.4 ^b	224.2 ± 32.7 ^a
Submucosa granulocytes	23.7 ± 2.9 ^b	36.6 ± 5.2 ^a	26.0 ± 2.4 ^b	37.6 ± 3.9 ^a	15.2 ± 2.1 ^b	20.5 ± 1.3 ^a	13.6 ± 1.2 ^b	19.2 ± 1.4 ^a
Lamina propria lymphoid cells	61.1 ± 7.7	54.5 ± 8.1	45.9 ± 4.6 ^b	68.5 ± 8.0 ^a	33.9 ± 2.7	34.6 ± 3.5	21.9 ± 2.7	24.7 ± 2.8
Lamina propria granulocytes	14.8 ± 1.7	16.34 ± 2.0	13.5 ± 1.4	15.8 ± 2.6	5.5 ± 0.4	5.7 ± 0.7	3.4 ± 0.6	3.7 ± 0.6
PCNA ⁺ cells total area (mm ²)	0.6 ± 0.2	0.6 ± 0.2	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
PCNA ⁺ cells total area/absorption area (%)	8.0 ± 2.5	7.0 ± 2.5	3.7 ± 0.8 ^b	7.1 ± 3.6 ^a	5.5 ± 2.9	5.8 ± 1.5	6.1 ± 3.0	6.9 ± 1.8

2 CTRL – fish fed the control diet, Blend – fish fed the blend diet. Values presented as mean ± SD (n = 10 samples per dietary treatment), based on quantitative analysis.

3 Independent samples t-test was employed to understand the significant differences between the study groups. Different letters denote significant differences between dietary

4 treatments within each intestinal section. PCNA, Proliferating cell nuclear antigen.

5 **Table 4.** Histomorphology of the different intestine sections of European seabass fed the control or the blend diets using a semi-quantitative analysis.

	Anterior Intestine		Mid Intestine		Posterior Intestine		Rectum	
	CTRL	Blend	CTRL	Blend	CTRL	Blend	CTRL	Blend
Villi length and integrity	1.9 ± 1.3	3.2 ± 2.0	2.4 ± 1.4	4.4 ± 2.5	2.1 ± 1.3	2.6 ± 1.2	1.4 ± 0.7	2.2 ± 1.2
Submucosa width	1.4 ± 0.5	2.0 ± 1.3	2.4 ± 1.0 ^b	4.3 ± 2.1 ^a	2.2 ± 0.7	3.1 ± 2.2	1.8 ± 0.8	2.4 ± 0.5
Lamina propria width	1.9 ± 0.9	3.2 ± 2.1	1.9 ± 0.9 ^b	3.1 ± 1.3 ^a	1.4 ± 0.7	1.4 ± 0.7	1.2 ± 0.4	1.2 ± 0.4
Submucosa lymphoid cells	2.3 ± 1.1	2.3 ± 1.1	2.6 ± 1.1 ^b	3.4 ± 0.7 ^a	2.2 ± 0.9	2.9 ± 0.9	2.4 ± 1.0 ^b	3.4 ± 0.7 ^a
Submucosa granulocytes	2.5 ± 1.2 ^b	3.5 ± 1.0 ^a	2.2 ± 0.9 ^b	3.6 ± 1.0 ^a	1.9 ± 0.9	2.7 ± 0.7	2.1 ± 0.7 ^b	2.8 ± 0.4 ^a
Lamina propria lymphoid cells	1.9 ± 0.6	2.2 ± 1.1	2.5 ± 0.7 ^b	3.4 ± 0.7 ^a	2.8 ± 1.0	3.1 ± 1.0	2.4 ± 0.8	2.5 ± 1.1
Lamina propria granulocytes	2.4 ± 1.0	3.4 ± 1.1	2.2 ± 0.8	2.3 ± 1.3	2.1 ± 0.7	2.4 ± 0.5	1.5 ± 0.5	1.4 ± 0.5

6 CTRL – fish fed the control diet, Blend – fish fed the blend diet. Scores 1-9 were assigned to villi length/integrity, submucosa width, and lamina propria width; while scores 1-5
7 were assigned to denote the presence of leucocytes (i.e., lymphoid cells and granulocytes) in the submucosa and lamina propria. Values presented as mean ± SD (n = 10
8 samples per dietary treatment), based on a semi-quantitative analysis. Independent samples t-test was employed to detect significant differences between the groups within
9 each intestinal section. Different letters denote significant differences between dietary treatment.

6.3.6 Quantitative vs. semi-quantitative approaches for evaluation of the intestinal health

The experimental diet-induced alterations of the intestine histomorphology were evaluated both quantitatively (Table 3) and semi-quantitatively (Table 4). In general, the differences captured by the quantitative assessment were also reflected in the semi-quantitative approach-based results, namely the increase in the submucosa thickness and lamina propria width, higher number of lymphoid cells in the submucosa and lamina propria, and granulocytes in the submucosa of the mid intestine of blend-fed fish. But some differences detected through the quantitative approach could not be perceived by the semi-quantitative analysis, namely the effects of the blend-diet on the villi length in the mid intestine, the increase in the lamina propria width in the anterior intestine and the rise in the number of granulocytes in the submucosa of the posterior intestine. Nevertheless, we observed a significant correlation between the variables of all the intestinal sections analysed quantitatively and semi-quantitatively, i.e., villi length, submucosa thickness, and lamina propria width, and presence of leucocytes in the submucosa and lamina propria (Supplementary Figure S4).

6.4 Discussion

The production and economic viability of the aquaculture sector are adversely impacted by the rising incidence of disease outbreaks in fish farms (Fernández Sánchez et al., 2022). Functional feeds are being recognised as promising tools to enhance fish robustness (Waagbø & Remø, 2020), a key factor for boosting the profitability of the aquaculture sector. Gaining an in-depth understanding of the morphology and functional potential of the gastrointestinal tract is crucial for comprehending the functionality of novel feed components and allow comparison of results among studies. The intestinal health of farmed fish is intimately related to both nutrition and immunity (Dawood, 2021), both of which can affect the morphology and functions of the intestine. To advance the field of fish intestinal health, we conducted a comprehensive characterisation of the European seabass intestine and propose specific indices and detailed scoring system that were correlated with intestinal functionality. In a previous study, Verdile et al. (2020b) performed a detailed examination of the intestine of rainbow trout (*Oncorhynchus mykiss*), exploring different sections in specimens of varying weight ranges. Although growth-induced modifications of the intestine's morphology were minimal, marked disparities were noted between the different sections of the intestine. Previous studies on European seabass intestine have only examined the anterior (Mota et al., 2023), posterior/rectal segments (Torrecillas et al., 2019), or both the anterior and posterior segments (Batista et al., 2020a). In contrast, our study provides a comprehensive analysis of the four distinct sections of the European seabass intestine – anterior, mid, posterior, and

rectum. The histomorphological parameters, gene expression patterns, and the position of the associated clusters in the PCA plot indicate the distinct morphology, functionality, and location of the different segments of the intestine. The samples collected from the anterior segment were linked to parameters associated with the processes of digestion and absorption. This segment exhibited the largest absorptive area and the longest villi, as well as the thinnest submucosa and highest count of neutral GC. In fish, the intestinal mucus layer produced by the GC is thought to play different roles, ranging from lubrication, absorption and immune defence (Leknes, 2010). The neutral GC, in specific, have been previously linked with improved digestion and absorption in European seabass (Batista et al., 2020a). The gene patterns observed in this segment further corroborate the importance of the anterior intestine within the digestive tract of fish. The feed efficiency of fish is highly dependent on both the digestive enzymes and the proteins involved in nutrient transportation processes (Takei & Loretz, 2010). In the present study, the involvement of anterior segment in the carbohydrates and lipid digestion and absorption is highlighted by the high expression of the genes encoding for the carbohydrate enzyme maltase (*malt*) and the fatty acid-binding protein 2 (*fabp2*) observed in this section. Therefore, the increased surface area observed in the anterior intestine in comparison with the other segments, coupled with the highest counts of neutral mucin-secreting cells, and high expression of genes that encode for digestive and transport proteins indicate that the region has the ideal environment for both nutrient digestion and absorption, in accordance with previous reports (Calduch-Giner et al., 2016).

The rectum and posterior intestine samples were found to be associated with immune-related variables. We observed the highest expression of immune-related genes in the rectum, followed, in general, by the posterior section. Moreover, the highest number of acid GC, that aid in protection against invading pathogens (Alesci et al., 2022; Kim & Khan, 2013), were found in the posterior/rectal sections, highlighting the importance of these segments for the intestinal immunity. A previous study examining in European seabass with a final body weight of 59 g revealed morphological differences between the posterior intestine and the rectum (Torrecillas et al., 2019). Our results show that the rectum of European seabass has longer villi compared to the posterior intestine and the posterior intestine has a higher density of GC, corroborating the findings of Torrecillas et al. (2019). In contrast to this study, we observed no variation in submucosa width between the posterior intestine and rectum, despite the latter having more lymphoid cells in the submucosa. These differences could be attributed to variations in fish body size (i.e., 60 vs 200 g in the present study) or exposure to different environmental challenges that might affect leucocyte infiltration. Our study compared four separate segments along the intestinal tract and revealed marked morphological differences in the rectum compared to the other three sections. Specifically, this region displayed the thickest muscularis, narrowest lamina propria, and longest microvilli. The thick muscularis

layer observed in rectum suggests that intestinal motility (Olsson & Holmgren, 2001) is crucial in this section, potentially for the release of waste. In addition, the longer microvilli of the rectum indicate that some important absorption processes still occur in this segment. When examining the gene expression patterns, it appears that the rectum is primarily involved in water absorption, rather than nutrient digestion, as compared to the other segments. As one of the main osmoregulatory organs in marine teleost, the intestinal epithelium plays a key role in water transport-processes through the action of membrane proteins, such as aquaporins (Breves et al., 2020). Similarly to findings reported by Giffard-Mena et al. (2007), the mid, posterior, and rectal segments of the intestine seem to be more relevant in the salt-water interactions across the intestinal barrier in European seabass, as evidenced by their high expression of *aqp1* compared to the anterior section.

The expression levels of the intestinal brush border membrane enzymes herein studied were segment-specific, except for alkaline phosphatase (*alp*). This enzyme displayed a consistent expression pattern across all the intestinal segments. The intestinal *alp* has been recognised for its role in preventing inflammation, both in the gut and at the systemic level (Lallès, 2020). According to the PCA plot, the variables *malt* and *fabp2* were found to be associated with the anterior section, along with other digestion/absorption-related parameters. In contrast, the expression of the *alp* gene appeared in the quadrants where the posterior and rectum samples clustered, in association with immune-related variables. Therefore, the ubiquitous expression of the *alp* gene throughout the intestine may play a role in maintaining intestinal homeostasis, which supports previous findings (Santos et al., 2022).

The selected gene expression patterns were unable to differentiate between the mid and posterior regions. Nonetheless, several histomorphometric indices, such as the absorption area, microvilli height, number of GC and acid GC, area of GC, and quantity of leucocytes in the submucosa and lamina propria, were able to distinguish these regions. While the posterior intestine samples were positioned in the same quadrant of the PCA biplot as the rectum samples, the mid intestine samples clustered in a specific area of the PCA biplot, and without any clear association with the assessed variables. The mid intestine seems, therefore, to be an intermediate region for both absorption and immune-related processes.

In order to understand the turnover of the enterocytes in different sections of the intestine, we analysed the localization and distribution of the proliferating cell nuclear antigen (PCNA) by immunostaining positive cells. The anterior portion of the intestine, followed by the rectum, had the strongest PCNA signalling, although there were differences in localization. Similar to what was observed in rainbow trout (Verdile et al., 2020b), the PCNA signal was detected both at the basis of the villi and at the villi branching points in the anterior intestine of European seabass, while in the rectum, PCNA immunolocalization was restricted to the base of the folds. The results suggest that the stem cell zone is most likely located at the basal part of the villi.

This is consistent with previous findings in rainbow trout by Verdile et al. (2020a), who found that the *sox9*⁺ cell zone (a marker for stem cells) in the intestine was similar to the PCNA⁺ cell zone. The understanding of the stem-cell population's localization in the gastrointestinal tract is crucial for the development of novel *in vitro* tools, such as organoids, for the study of fish intestine. Organoids have recently emerged as one of the most promising tools for studying the intestinal health of farmed animals (Beaumont et al., 2021).

Fish nutrition studies should accurately report the impacts of feeds on the intestinal health, and for this it is important to precisely describe the changes in the intestine segment selected for the analysis. Based on our findings, we propose that the intestine of European seabass can be divided into four different segments with unique morphological and/or gene expression profiles: 1) the anterior intestine, 2) the mid intestine, 3) the posterior intestine, and 4) the rectum. It is important to note that the length of the intestine can vary with fish size, and therefore, we recommend a standardized method for identifying and sampling these segments. Specifically, the anterior intestine should be sampled immediately after the pyloric ceca; the mid intestine should be sampled from the middle region between the pyloric ceca and the ileorectal valve, the posterior intestine should be the portion preceding the ileorectal valve, and the rectum should be the segment immediately after the ileorectal valve. Using this standardized approach for sample collection and nomenclature for intestine segments will ensure consistent and accurate reporting of feed impacts on intestinal health and allow comparison with future studies.

After conducting a detailed study on the different intestinal segments of European seabass, we aimed to investigate the effects of a novel macro- and microalgae blend on the intestinal functionality of the fish. Although the dietary inclusion of the blend did not affect fish growth performance or feed utilization parameters, the results suggest that the diet can alter the intestine morphology. Most studies that evaluated fish feed utilization and immune status relied either on the anterior intestinal segment (Batista et al., 2020a) or on the posterior/rectal segment (Torrecillas et al., 2019), respectively. However, in this study, the subtle differences between the CTRL and the Blend diet were mostly perceived in the mid intestine. When the impact of the blend was evaluated in the different intestinal segments using either a quantitative or semi-quantitative approach, the mid section presented, in general, the highest response towards the diet, which may indicate that this segment is more sensitive to external stimuli. The feeding trial lasted for only eight weeks, hence the observed villi shortening and the reduced number of acid GC in the mid intestine are likely to be an adaptive response to the diet. Further evaluation of such alterations in the intestine structure is required after a longer-term feeding trial. Nonetheless, some immunomodulatory effects were already observed in various intestinal sections, including thickening of the submucosa (mid intestine), widening of the lamina propria (anterior and mid sections), an increase in the number of

lymphoid cells in the submucosa (mid and rectum segments), granulocytes in the submucosa, and lymphoid cells in the lamina propria of the mid intestine. These observations, along with the increased expression of immune- and oxidative stress-related genes (*il-8*, *pisc1* and *gpx*), in the mid region, point towards an immunomodulatory effect promoted by the algae blend. This potential functionality should be further explored after a disease challenge, such as a bacterial infection. However, it is important to note that experimental conditions, namely temperature and salinity, can influence gut structure and functionality and yield varying outcomes (Cabillon & Lazado, 2019; Kokou et al., 2019; Liu et al., 2022b). In a previous study, we observed that European seabass fed a micro- and macroalgae blend and reared under similar temperature conditions as the present trial (21 °C), but with higher salinity levels (35‰), had an enhanced mucosal immune response upon infection with *Tenacibaculum maritimum* (Ferreira et al., 2023b). Overall, these results suggest that dietary supplementation with algae could be a promising strategy to improve the robustness of European seabass.

To evaluate the impact of feeds on fish intestinal health, researchers have often conducted a semi-quantitative/score-based analysis focused on a determined intestinal segment. Such scoring system commonly used in literature often lacks clear definition and description of the selected indices and can lead to unreproducible results (Penn et al., 2011; Silva et al., 2015). Moreover, due to the lack of a well-defined and validated scoring system, it is more likely for untrained individuals to make errors and have reproducible results (Vatsos, 2021). As morphologic traits vary throughout the tract, it is crucial to assess each section's specific morphologic traits and all samples beforehand to ensure the accurate assignment of the score. Hence, our study provides a detailed description of a set of scores for the semi-quantitative assessment of several morphological parameters of the intestine such as 1) villi length and integrity; 2) submucosa thickness; 3) lamina propria width; 4) submucosa lymphoid cells; 5) submucosa granulocytes; 6) lamina propria lymphoid cells; and 7) lamina propria granulocytes. The proposed scoring system includes validated and clearly defined criteria for each intestinal section, allowing researchers to select a scoring method that generates reliable and consistent data. Alternatively, recent studies investigating the morphology of the intestine have utilized quantitative approaches to produce precise measurements (Batista et al., 2020a; Torrecillas et al., 2019; Vatsos, 2021). In the present work, we have also developed a detailed description of the indices and methods used for the quantitative assessment, which also allow direct comparisons among intestinal segments. We have used an image analysis software to automatically estimate the absorptive area, count the GC, and evaluate cellular proliferation (determined by the total area occupied by PCNA⁺ cells). For all other parameters, we have employed a semi-automatic approach. The quantitative assessment method enables us to measure the same parameters that are commonly evaluated through a score-based analysis (e.g., villi length, submucosa and lamina propria widths, and leucocyte infiltration) (Penn et

al., 2011; Silva et al., 2015; Urán et al., 2008; Vatsos, 2021), as well as several other additional parameters such as total absorptive area, muscularis thickness, and total GC counts in a cross-section. This approach has enormous potential to further evolve into a completely reliable automatic image analysis using artificial intelligence technologies for fast-track evaluation of fish intestinal health.

Although the semi-quantitative analysis is a less time-consuming and relatively easier method to assess the intestine histomorphology, it relies on pre-defined scores, which can increase bias (Meyerholz & Beck, 2019). In addition, unlike the quantitative assessment, the semi-quantitative analysis requires highly trained and experienced observers (Vatsos, 2021). In the present study, we found a significant correlation between the values obtained by both quantitative and semi-quantitative analyses for the parameters evaluated within each intestinal segment. This indicates that both methods are valid for assessing intestinal health. While the semi-quantitative analysis may be useful for a quick pre-assessment, it is important to define the scoring system correctly to ensure good repeatability. However, we prefer the quantitative approach overall, as it allows for more easily comparable measurements across studies. To ensure robust and reproducible data from both methods, it is crucial that studies provide a detailed description of the methodology for each index in the methodology section.

In conclusion, by providing an in-depth characterisation of the different segments of the intestine (i.e., anterior, mid, posterior, and rectal) of European seabass, the present study provides valuable information about the intestinal morphology. Overall, we found that histological characteristics and gene expression profiles were specific to each intestinal segment. The anterior region appears to be primarily associated with absorption processes, with the largest absorptive area, longest villi, and a high expression of genes encoding digestive and transport proteins. In contrast, the rectum, followed by the posterior intestine, displayed the highest expression values for immune-related genes, highlighting their importance for mucosal immunity. The thick muscularis and long microvilli observed in rectum, coupled the high expression of the *aqp1* gene, most likely indicates that both waste release and water absorption are also important processes occurring in this segment. Finally, the mid intestine appears to be a transitional region for both absorption and immunity. Hence, the generated knowledge can assist in arriving at better decisions regarding the choice of the appropriate segment and morphometric indices for future experiments to study nutritional modulation and its effects on intestinal health. Furthermore, transition from a semi-quantitative to a quantitative analysis of intestine histomorphology is crucial for adopting digital technologies that produce more precise and fast histology-based results. As a futuristic perspective, the quantitative analysis offers a promising avenue for machine learning approaches in this field, allowing for high throughput image analysis of intestinal histological sections using artificial intelligence.

6.5 Acknowledgments

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6.7 Authors contributions statement

M.F. carried out the methodology, investigation, formal analysis and writing of the manuscript draft. V.S. and B.O. were involved in the methodology, investigation, formal analysis, and writing – review and editing. A.C.-S. participated in methodology and writing – review and editing. H.A. and J.D. participated in the research conceptualization and writing – review and editing. K.V. was involved in the supervision and writing – review and editing. L.M.P.V. participated in funding acquisition, research conceptualization, supervision, validation, and writing – review and editing. All authors discussed the results, contributed to the article, and approved the submitted version.

6.8 Competing interests

H.A. and J.D. were employed by the companies ALGAplus and SPAROS, respectively. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6.9 Supplementary material

Supplementary Table S1. Description of the parameters used to evaluate the intestinal morphology of European seabass juveniles – quantitative analysis.

Parameter	Description
Cross-sectional perimeter (mm)	The cross-sectional perimeter was automatically determined by the software after encircling the cross section using the commands "Measure" -> "Measurement and ROI" -> "Arbitrary line".
Absorption area (mm²)	The total area occupied by the villi – absorption area - was determined automatically by the software. Firstly, a polygon was drawn from the base of the villi, right after the submucosa, using the commands "Automatically create ROI" -> "Closed Polygon". Then, the software automatically determined the area occupied by the villi based on color, using the following commands: "Measure" -> "Count and Measure" -> "Manual HSV Threshold" -> "New Threshold" (select the tissue area with the tool) -> "Count and Measure".
Muscularis thickness (μm)	The muscularis was measured from the outer layer of the section until the outer layer of the submucosa, in eight regions along the section, using the following commands: "Measure" -> "Measurement and ROI" -> "Arbitrary line". The average muscularis thickness per section was determined.
Submucosa width (μm)	The submucosa width was measured from the outer to the inside limits of the submucosa layer, in eight regions along the section, using the commands "Measure" -> "Measurement and ROI" -> "Arbitrary line". The average submucosa width per section was determined.
Lamina propria width (μm)	In eight villi selected for the villi length measurement, three measurements of the lamina propria width were made along the villi (one at the base, one in the middle and one at apex of the villi), using the commands "Measure" -> "Measurement and ROI" -> "Arbitrary line". The average lamina propria width per section was determined.
Villi length (μm)	The eight highest villi per section were selected for the measurement. The villi length was measured from the tip of the villi to the apex, using the following commands: "Measure" -> "Measurement and ROI" -> "Polyline". The average villi length of per section was determined.
Microvilli height (μm)	In the eight villi selected for the villi length measurement, eight measurements of the microvilli height were made along the villi, using the commands "Measure" -> "Measurement and ROI" -> "Arbitrary line". The average microvilli height per section was determined.
Acid GC	Based on the color of the cells (blue), the number of acid GC was automatically determined by the software, using the following commands: "Measure" -> "Count and Measure" -> "Manual HSV Threshold" -> "New Threshold" (select the blue cells with the tool) -> "Count and Measure".
Neutral GC	Based on the color of the cells (magenta), the number of neutral GC was automatically determined by the software, using the following commands: "Measure" -> "Count and Measure" -> "Manual HSV Threshold" -> "New Threshold" (select the magenta cells with the tool) -> "Count and Measure".
Goblet cells (GC)	Sum of the acid GC and neutral GC.
Average area GC (μm²)	After automatic counting of the GC, the average area of the cells is provided by the software.
Submucosa lymphoid cells	Six photos of the submucosa were randomly taken at 400 x magnification. All lymphoid cells present in those photos were counted and an average number of lymphoid cells per section was determined.
Submucosa granulocytes	Six photos of the submucosa were randomly taken at 400 x magnification. All granulocytes present in those photos were counted and an average number of granulocytes per section was determined.
Lamina propria lymphoid cells	Six photos of the lamina propria were randomly taken at 400 x magnification. All lymphoid cells present in those photos were counted and an average number of lymphoid cells per section was determined.
Lamina propria granulocytes	Six photos of the lamina propria were randomly taken at 400 x magnification. All granulocytes present in those photos were counted and an average number of granulocytes per section was determined.
PCNA⁺ cells total area	The PCNA ⁺ cells (immunohistochemistry) were automatically detected by the software based on their color (brown), using the following commands: "Measure" -> "Count and Measure" -> "Manual HSV Threshold" -> "New Threshold" (select the brown cells with the tool) -> "Count and Measure".

All measurements were performed using the imaging software Olympus cellSens Standard 2.2.

Supplementary Table S2. Primers used in the RT-PCR analysis and corresponding annealing temperature (°C), efficiency of the RT-PCR (%), accession number (GenBank ID) and reference.

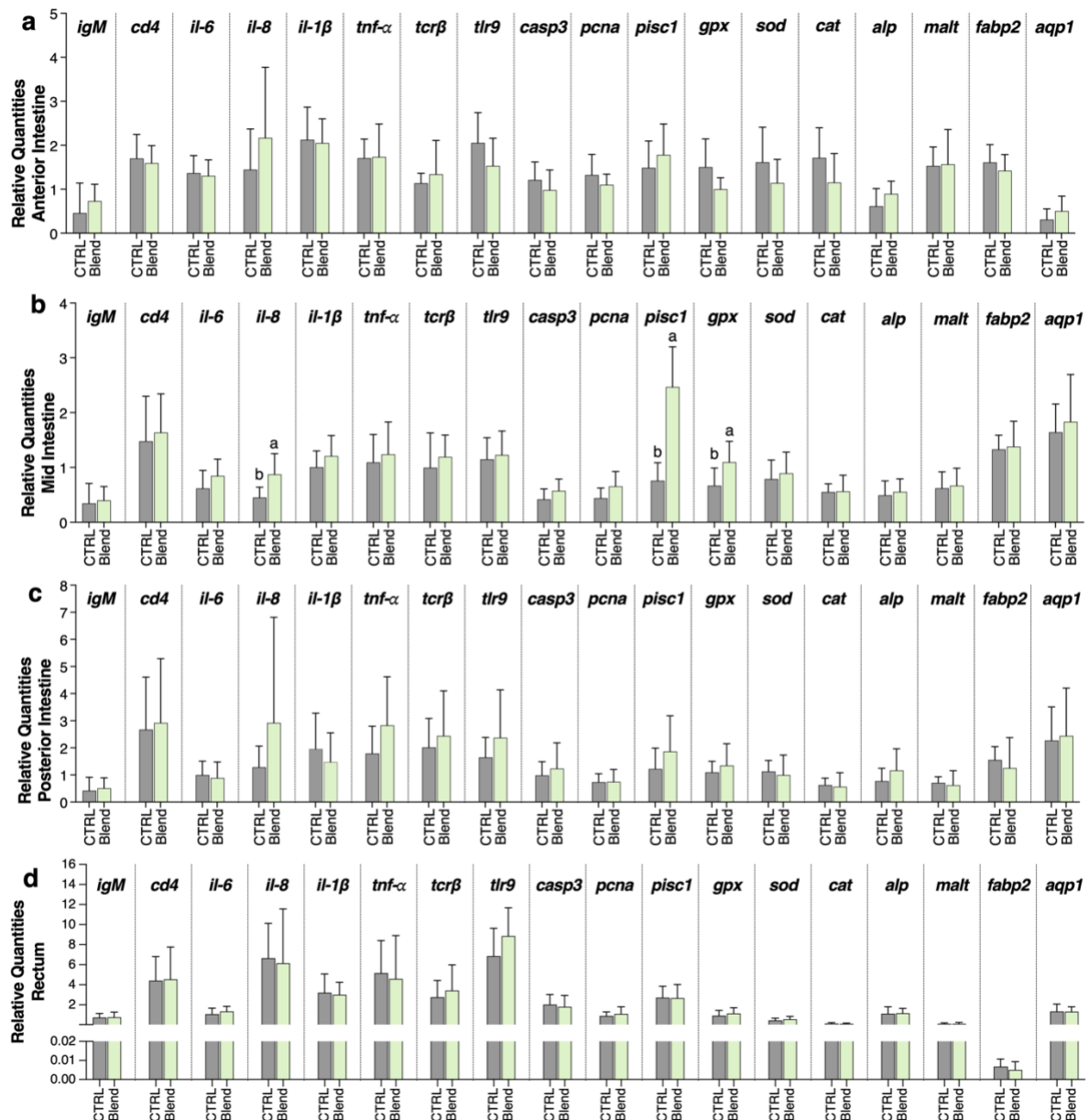
Gene	Primer Forward	Primer Reverse	Annealing T (°C)	Efficiency (%)	GenBank ID	Reference
<i>18s</i>	CCAACGAGCTGCTGACC	CCGTTACCCGTGGTCC	58	100	AY831388.1	Núñez-Ortiz et al. (2016)
<i>β-actin</i>	CAAAGCCAACAGGGAGAAGATGA	ACCGGAGTCCATGACGATAC	60	102	AJ537421	Couto et al. (2015)
<i>ef1α</i>	AACTTCAACGCCAGGTCAT	CTTCTTGCCAGAACGACGGT	58	101	AJ866727.1	Azeredo et al. (2017a)
<i>gapdh</i>	CAAGGTTGAGGGTGACAAGC	CAGCCTCACCCATTGATG	60	102	AY863148	Couto et al. (2015)
<i>igM</i>	AGAGGAGCTGCAGGAGGACA	GGACCTCCAGGCTGTGTGAC	60	105	AJ400233	Couto et al. (2015)
<i>cd4</i>	ACTTGTGATTGGGCAGTCC	TGATGAGATGAGTGGGGTGA	60	102	AM849812	Couto et al. (2015)
<i>il-6</i>	AGGCACAGAGAACACGTCAA	AAAAGGGTCAGGGCTGTCTG	58	103	AM490062.1	Machado et al. (2021)
<i>il-8</i>	CGCTGCATCCAAACAGAGAGCAAAC	TCGGGGTCCAGGCAAACTCTT	58	100	AM490063	Machado et al. (2018)
<i>il-1β</i>	AGCGACATGGTGCGATTCT	CTCCTCTGCTGTGCTGATGT	58	94	AJ269472	Azeredo et al. (2017b)
<i>tnf-α</i>	AGCCACAGGATCTGGAGCTA	GTCCGCTTCTGTAGCTGTCC	60	107	DQ070246	Machado et al. (2018)
<i>tcrβ</i>	GACGGACGAAGCTGCCCA	TGGCAGCCTGTGTGATCTTCA	58	106	AJ493441	Torrecillas et al. (2015)
<i>tlr9</i>	TCTTGGTTTGGCCACTTCTTGCGT	TACTGTTGCCCTGTTGGGACTCTGG	60	107	KX399289	Byadgi et al. (2019)
<i>casp3</i>	TGATGTCGTCTCTGCCGTAG	ACCACCTCATACGCATCCTC	60	104	DQ345773	Couto et al. (2015)
<i>pcna</i>	TGAAGTGTGCAGGAAACGAAGA	GGCGAGTGTGTCTGCATTGT	60	106	JQ755266	Rawling et al. (2019)
<i>pisc1</i>	TCGTCCTCATGGCTGAACC	GCGGTTAAAGCGTGATATTG	60	105	MT066191	Barroso et al. (2020)
<i>gpx</i>	GTTTGGACATCAGGAGAACTGC	CATCGCTGGGGTATGGAAGC	58	102	DT044993	Azeredo et al. (2017a)
<i>sod</i>	GGAGAGTGATTACAGCCCCTG	GGAAACCATGCTCACCAGGA	58	102	CX660893.1	Machado et al. (2021)
<i>cat</i>	GAGGTTTGCCTGATGGCTAC	TGCAGTAGAAACGCTCACA	60	108	FJ860003	Morcillo et al. (2015)
<i>alp</i>	TTACCTCTGTGGGGTCAAGG	TAGCCCATTTGAGGATGGAG	60	94	FJ860000	Couto et al. (2015)
<i>malt</i>	ATGCCATGGAGGTGACTTTC	CAACCATTTCAGGCGTAGGT	60	105	AM419039	Couto et al. (2015)
<i>fabp2</i>	CCGCAACGACAACTATGATAAG	TGGACTCTTTGATGTGAACTTG	60	106	MF566099	Kaitetzidou et al. (2015)
<i>aqp1</i>	CAAGGCAGTCATGTATATTG	AGAGAGTTGAGCCCCAGT	60	105	DQ924529	Giffard-Mena et al. (2011)

18s, 18S rRNA; *β-actin*, Beta actin; *ef1α*, Elongation factor 1 alpha; *gapdh*, Glyceraldehyde-3-phosphate dehydrogenase; *igM*, Immunoglobulin M; *cd4*, Cluster of differentiation 4; *il-6*, Interleukin-6; *il-8*, Interleukin-8; *il-1β*, Interleukin-1 beta; *tnf-α*, Tumor necrosis factor-alpha; *tcrβ*, T-cell receptor antigen receptor beta chain; *tlr9*, Toll-like receptor 9; *casp3*, Caspase 3; *pcna*, Proliferating cell nuclear antigen; *pisc1*, Piscidine 1; *alp*, Alkaline phosphatase; *malt*, Maltase; *fabp2*, Intestinal fatty acid binding protein; *aqp1*, Aquaporin 1; *gpx*, Glutathione peroxidase; *sod*, Superoxide dismutase; *cat*, Catalase.

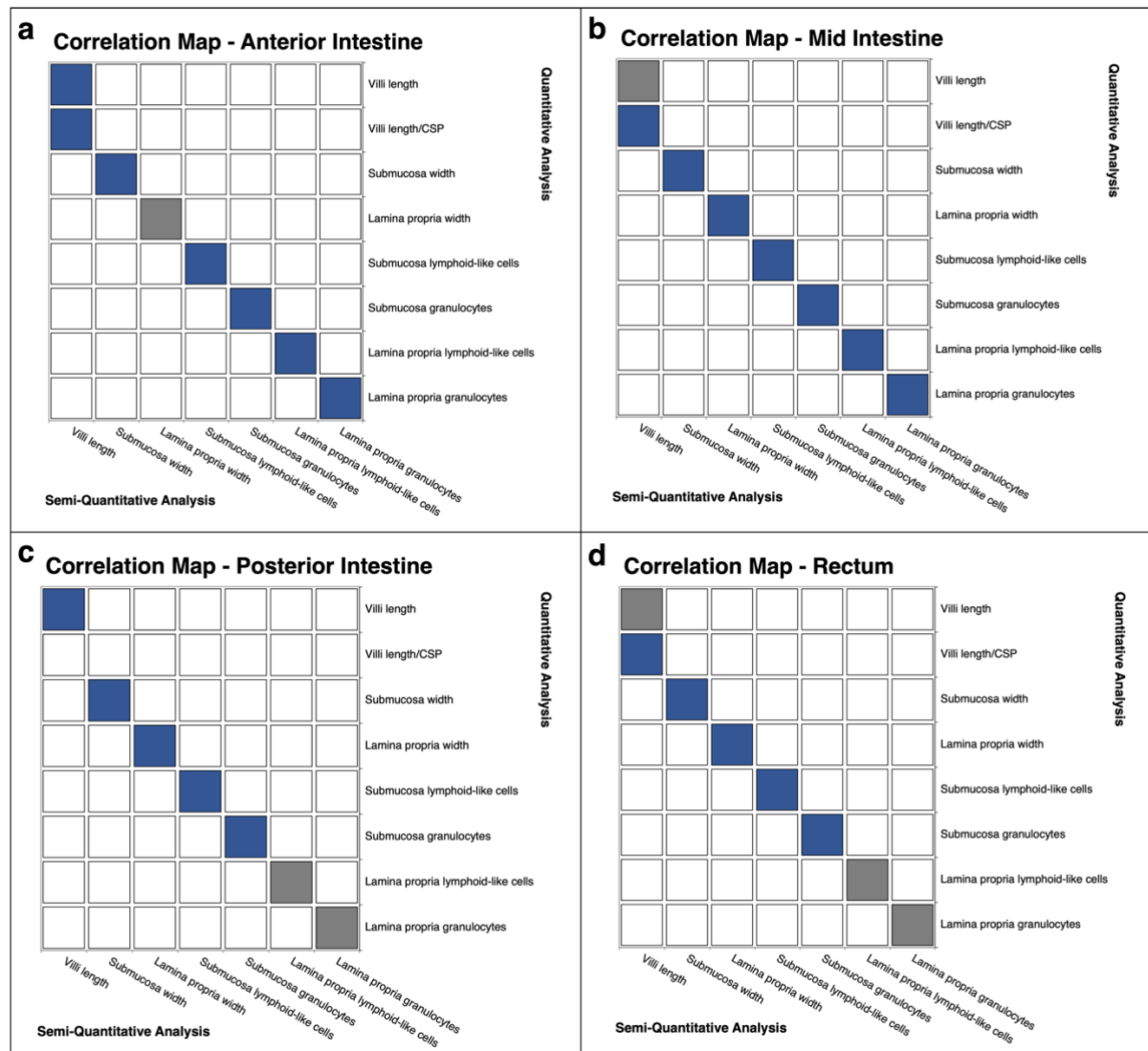
Supplementary Table S3. Histomorphology of the different intestine sections of European seabass using a semi-quantitative analysis.

	Intestine Sections			
	Anterior	Mid	Posterior	Rectum
Villi length and integrity	2.4 ± 1.8	3.0 ± 2.2	2.6 ± 1.2	1.8 ± 1.1
Submucosa width	1.7 ± 1.0	3.0 ± 1.8	2.6 ± 1.1	2.0 ± 0.7
Lamina propria width	2.6 ± 1.7	2.5 ± 1.2	1.4 ± 0.7	1.2 ± 0.4
Submucosa lymphoid cells	2.3 ± 1.0	3.0 ± 1.0	2.5 ± 0.9	2.8 ± 1.0
Submucosa granulocytes	2.8 ± 1.2	2.7 ± 1.2	2.3 ± 0.9	2.5 ± 0.7
Lamina propria lymphoid cells	2.1 ± 0.0	2.5 ± 0.8	2.5 ± 1.0	2.0 ± 0.9
Lamina propria granulocytes	2.5 ± 1.1	2.3 ± 1.1	2.3 ± 0.6	1.5 ± 0.5

Scores 1-9 were assigned to villi length/integrity, submucosa width, and lamina propria width; while scores 1-5 were assigned to denote the presence of leucocytes (i.e., lymphoid cells and granulocytes) in the submucosa and lamina propria. Values presented as mean ± SD (n = 20 samples per section), based on a semi-quantitative analysis.



Supplementary Figure S2. Genes expression in the different sections of the intestine of European seabass juveniles fed the control or the blend diets. a) Anterior intestine; b) Mid intestine; c) Posterior intestine; and c) Rectum. Relative quantities of immune-related genes (*igM*, *cd4*, *il-6*, *il-8*, *il-1β*, *tnf-α*, *tcrcβ*, *tlr9*, *casp3*, *pcna* and *pisc1*); genes associated with oxidative stress (*gpx*, *sod*, and *cat*); and genes related to nutrient digestion and absorption (*alp*, *malt*, *fabp2*, and *aqp1*) are presented as mean ± SD (n = 16 samples per dietary treatment). Independent samples t-test was employed to understand the significant differences between the study groups. Significant differences are detected only for the mid intestine, with different letters denoting significant differences between dietary treatments.



Supplementary Figure S4. Correlogram showing the correlation between the equivalent histomorphometric variables measured by quantitative and semi-quantitative analyses of the in the anterior (a), mid (b), posterior (c), and rectal (d) sections of the intestine of European seabass. Correlations with statistical significance at $P < 0.05$ are displayed as blue and with statistical significance at $P < 0.1$ are displayed as grey.

Chapter 7.

Optimised protocols for isolation of immune cells from haematopoietic and mucosal organs of European seabass

By

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In preparation for Publication

Abstract

The development of *in vitro* tools for aquaculture is crucial for advancing our understanding of immune responses and immunonutrition in economically significant farmed fish species, such as the European seabass. The goal of this study was to optimize the protocol for isolating and culturing leucocytes from the head-kidney and posterior intestine of European seabass. The results revealed that the 60%/40% gradient was ineffective in separating erythrocytes. Following density separation (34%/51% for the head-kidney and 25%/75% for the intestine), cell morphology showed reduced erythrocyte presence and an enrichment of lymphocyte-like and monocyte/macrophage-like populations. While a high yield of viable leucocytes was achieved from the head-kidney, further refinement is needed for the intestine isolation protocol. Notably, the use of lectins for immunophenotyping did not effectively distinguish distinct cell populations in the head-kidney and intestine of European seabass. Subsequent isolation and culturing of immune cells revealed their attachment capabilities, and functional assays confirmed their phagocytic activity. These findings suggest that the adherent cells exhibit properties inherent to macrophage-like cells. This study provides valuable insights into immune cell isolation from the head-kidney and intestine of European seabass, representing a critical initial step in developing innovative *in vitro* tools for the study of immunity and immunonutrition in aquaculture.

7.1 Introduction

The development of *in vitro* tools for aquaculture is increasingly crucial due to their potential to significantly advance our comprehension of immune responses and immunonutrition in economically significant farmed fish species (Rocha et al., 2023). Notably, the European seabass, a species highly desirable in the aquaculture sector, has garnered attention as a key marine fish model (Vandeputte et al., 2019). As the global population continues to expand, the demand for nutritious protein sources, particularly fish, is on the rise. However, the intensification of aquaculture practices, especially for economically important fish species like the European seabass, has resulted in disease outbreaks (Boyd et al., 2020; Fernández Sánchez et al., 2022). Moreover, there is a rising concern regarding antimicrobial resistance. Consequently, the European Union has enforced stringent regulations governing the application of antibiotics in food production (Ferri et al., 2017; Lulijwa et al., 2020).

To address these challenges, the aquaculture industry is exploring innovative feed formulations aimed at enhancing fish robustness without reliance on antibiotics (Kiron, 2012). The welfare of fish relies heavily on the optimal functioning of haematopoietic organs, including the head-kidney, as well as mucosa-associated lymphoid tissues (MALTs). Within the MALTs, gut-associated lymphoid tissues (GALTs) play a crucial role in maintaining host homeostasis. However, the limited knowledge of the intestinal immune system in fish in comparison to mammals presents an obstacle to the development of efficient tools to fortify fish immunity through nutritional strategies (Guardiola et al., 2022; Kiron, 2012; Salinas et al., 2022). The evaluation of new feed compounds for aquaculture still predominantly relies on a substantial number of animal trials. Therefore, the development of efficient *in vitro* tools, capable of mimicking *in vivo* outcomes, is essential to acquire crucial insights into the fish immune system and the role of immunonutrition for the long-term sustainability of the aquaculture sector (Goswami et al., 2022). These tools hold the potential to revolutionize disease management and nutrition strategies in the aquaculture industry, enhancing its sustainability and productivity (Goswami et al., 2023).

The progress of the research on the fish immune cells is severely compromised by the lack of adequate cell markers and the complex isolation protocols. In the case of head-kidney leucocytes, several studies have employed them as a tool to assess the impact of environmental contaminants or nutrients on immune responses (Azeredo et al., 2017b; Machado et al., 2021; Morcillo et al., 2015). Isolation protocols for intestinal cells of fish have been described for some fish species such as rainbow trout (*Oncorhynchus mykiss*) (Bernard et al., 2006), gilthead seabream (*Sparus aurata*) (Salinas et al., 2007) and Atlantic salmon (*Salmo salar*) (Park et al., 2020b). In European seabass, the GALT harbours

different cell populations, namely T and B cells, macrophages and granulocytes (Picchiatti et al., 2021b). Previous studies have shown that European seabass GALT is associated with the expression of marker genes for T cells (i.e., *rag-1*, *cd3ε*, *tcrlβ*, *cd8α*, and *cd4*) (Boschi et al., 2011; Picchiatti et al., 2021a). Nevertheless, there is no in-depth knowledge of the characteristics and functions of the different immune cell populations present in the European seabass intestine.

Taking all these factors into account, the present study aimed to optimize the isolation process for immune cells from both the head-kidney and intestine of the European seabass. The ultimate goal was to comprehensively characterize these isolated cell populations through morphological, cytometric, and functional assays. The findings of the present study are expected to enhance our understanding of fish health, immunonutrition, and immunity, with the potential to significantly impact the aquaculture industry, making it more sustainable and productive.

7.2 Materials and methods

7.2.1 Ethical statement

The fish employed in this study were sourced from continuous experimental trials, which had received approval from the animal welfare committee of the Interdisciplinary Centre for Marine and Environmental Research (CIIMAR, Matosinhos, Portugal) - ORBEA-CIIMAR - and were conducted in accordance with the ARRIVE guidelines. The research was closely monitored by the National Veterinary Authority (DGAV, Portugal), ensuring full adherence to both European Union guidelines (Directive 2010/63/EU) and Portuguese legislation (Decreto-Lei no. 113/2013, de 7 de Agosto) on the protection of animals utilized for scientific purposes.

7.2.2 Experimental conditions and fish sampling

European seabass juveniles were housed in a saltwater recirculation system at CIIMAR's Bioterium of Aquatic Organisms (BOGA) with controlled environmental conditions, including a temperature of 20°C, dissolved oxygen saturation of 8 ppm, and a 12-hour light and 12-hour dark cycle. Fish were fed a commercial feed (AQUASOJA, Portugal) containing 50% crude protein and 20% crude fat based on dry matter, with feeding occurring twice a day. Before tissue samples were collected, the fish underwent a 48-hour fasting period. Subsequently, they were anesthetized using 2-phenoxyethanol (Sigma-Aldrich, USA), individually weighed, and had all blood removed from the caudal vein. To ensure humane euthanasia, their spinal cords were severed, and the fish were thoroughly cleaned with a 70% ethanol solution. For tissue sampling, the focus was on the head-kidney and the

posterior intestine. The head-kidney was carefully removed and promptly placed in a cell isolation medium (Leibovitz's L-15 Media, Sigma-Aldrich) containing 2% foetal bovine serum (FBS, Sigma-Aldrich) and 50 µg/mL penicillin/streptomycin. In the case of the posterior intestine, the dissected tissue was rinsed with phosphate buffer saline (PBS, Sigma-Aldrich), opened longitudinally, and cut into approximately 1 cm long fragments. All procedures were performed at room temperature (RT). Note that, starting from this point onward, all PBS solutions used have been adjusted to an osmolality of 370 mOsm.

7.2.3 Leucocytes isolation from the head-kidney

In this study, the immune cell isolation protocols for the head-kidney were adapted from the work of Azeredo et al. (2017b), Machado et al. (2021) and Park et al. (2020a). Each head-kidney was mechanically dissociated using a 40 µm nylon cell strainer and a syringe plunger. The harvested cells were then washed with L-15 medium through centrifugation at 500 × g for 5 minutes at 4 °C. It should be noted that all subsequent washing steps were carried out under the same centrifugation conditions (500 × g, 5 minutes, 4 °C).

To optimize the cell isolation process from the head-kidney of European seabass and achieve a high yield of viable leucocytes, experiments were conducted. This optimization involved the evaluation of two gradients of continuous Percoll solutions (Sigma-Aldrich), which were utilized to separate leucocytes from erythrocytes. Percoll solutions at concentrations of 34%, 51%, 40%, and 60% were prepared by diluting the stock solution with PBS 10x and the following Percoll gradients were subsequently generated: 34/51% and 40/60%. Following centrifugation of the harvested cells, the resulting pellet was resuspended in L-15 medium. For both Percoll gradients, 6 mL of head-kidney cell suspension was carefully layered on 20 mL of the prepared gradients. The samples were then centrifuged at 500 × g for 40 minutes at 4 °C, and the interface between the two Percoll gradients was collected and washed twice with L-15 medium.

7.2.4 Leucocytes isolation from the posterior intestine

In this study, the immune cell isolation protocols for the posterior intestine were adapted from the work of Salinas et al. (2007) and Park et al. (2020b). The posterior intestine fragments were initially transferred to tubes containing a solution of dithiothreitol (DTT) at a concentration of 0.145 mg/mL, which was supplemented with 5 mM EDTA (VWR). The tissue was incubated at RT for 20 minutes in the DTT solution, with gentle mixing every five minutes. The tissue fragments were then transferred to a tube containing L-15 medium and gently inverted to remove the DTT, ensuring efficient subsequent action of collagenase. After the wash, the intestine fragments were placed in tubes with a solution of collagenase

IV (Gibco) at a concentration of 0.37 mg/mL. Various treatment conditions were tested during this step, including incubation with collagenase IV for 30 minutes and 2 hours, with and without agitation at 700 rpm (Cole-Parmer, USA). The enzymatically treated posterior intestine tissues were then mechanically pushed through a 100 µm nylon cell strainer using a syringe plunger. The remaining DTT, L-15 washing medium, and collagenase solutions were also filtered through the cell strainer to recover leucocytes that might have remained in suspension. The harvested cells were washed with L-15 medium.

The cell isolation protocol was optimized to specifically isolate the posterior intestine leucocytes by testing single-step and double-step separations using a continuous Percoll gradient. In the single-step separation, 6 mL of posterior intestine cell suspension was carefully layered onto 20 mL of the prepared 25%/75% continuous Percoll gradient. The samples were then centrifuged at $500 \times g$ for 40 minutes at 4°C, and the interface between the two Percoll gradients was collected and washed twice with L-15 medium. In the double-step separation, the interphase obtained from the first gradient was recovered as previously stated. This suspension was then washed with L-15 medium, layered once again on 20 mL of a 25%/75% Percoll gradient, and centrifuged once again at the same conditions. The interface between the two Percoll gradients was collected and washed twice with L-15 medium. The isolated cells were then resuspended in L-15 GlutaMAX medium (Sigma-Aldrich), which was supplemented with 10% FBS.

7.2.5 Flow cytometry analysis and sorting

To characterize the various leucocyte populations present in the head-kidney and posterior intestine of European seabass, and to assess the possible lectin binding selectivity of these populations, fluorescence-activated cell sorting (FACS) was employed. Following the isolation of head-kidney and posterior intestine cells, the cell suspensions were washed with PBS and adjusted to a concentration of 1×10^6 cells. Subsequently, these samples underwent staining with different dyes, either individually or in combination: fixable viability dye (FVD) labelled eFluor 780 (Invitrogen, USA), wheat germ agglutinin (WGA) labelled with fluorescein (Vector Laboratories, USA), and *Lycopersicon esculentum* (tomato) lectin (LEL) labelled with DyLight 649 (Vector Laboratories), during a 30-minute incubation at RT in the dark.

Flow cytometry analysis was conducted using a BD FACSAria II Cell Sorter, which was equipped with a 488 nm blue laser, a 561 yellow-green laser, and a 633 nm red laser (BD Biosciences, USA). Parameters such as forward scatter (FSC), side scatter (SSC), and fluorescence channels corresponding to the fluorophores used were evaluated. Subsequently, hypothetical different cell populations were sorted into 5 mL tubes containing

L-15 medium. The BD FACSDiva Software (BD Biosciences) was employed for compensation and data analysis.

7.2.6 Culturing of leucocytes from the head-kidney and posterior intestine

The cell culturing protocol was devised to maximize the yield of adherent cells and obtain an enriched population of macrophage-like cells derived from both the head-kidney and posterior intestine of European seabass. Following the isolation process, head-kidney and posterior intestine cells were cultured on 96-well cell culture plates (Nunc MicroWell, Thermo Scientific) and incubated at 23 °C. After a two-hour incubation, the non-adherent cells were removed by carefully aspirating the medium from the wells, followed by two rinsing steps using warm L-15 medium. Subsequently, the wells were replenished with fresh L-15 GlutaMAX medium supplemented with 10% FBS, and the plates were incubated for 24 hours at 23 °C.

7.2.7 Detachment of adherent cells

The recovery of primary adherent cells was enhanced through the implementation of a cell detachment process. After a 24 hours incubation period, non-adherent cells were removed through three rinsing steps using warm L-15 medium. Following this, ice-cold detachment solutions were introduced to the wells, with two different solutions tested: macrophage detachment solution DXF (PromoCell, USA) and PBS-EDTA 5 mM. The plates were subsequently placed on ice for 40 minutes to facilitate the detachment of cells. The cells were then harvested, and the surfaces of the cell culture plates underwent a thorough rinse with L-15 medium to maximize the yield of recovery. An inverted microscope was utilized to confirm cell detachment on the culture plates. The collected cells were washed twice with L-15 medium through centrifugation.

7.2.8 Cell viability and morphology analysis

Following the isolation, culturing, and detachment procedures, cell viability was determined through the trypan blue exclusion assay. The obtained cell suspensions underwent centrifugation at $500 \times g$ for 5 minutes, and the resulting pellet was re-suspended in PBS. This suspension was then combined with a trypan blue solution (Sigma-Aldrich) in a 1:1 proportion (v/v). Viable and non-viable cells were quantified using a haemocytometer beneath an optical microscope.

To assess the cellular morphology, cytospinning at 1000 rpm for 5 minutes was employed using the Shandon CytoSpin III. Following centrifugation, the cells were fixed with ice-cold methanol for 5 minutes and subsequently subjected to Diff-Quick staining – being stained

firstly with eosinophilic stain for 2 minutes, followed by being stained with basophilic stain for 3 minutes. The cells were observed using a light microscope (Olympus BX51, Germany), and the images were captured by a camera (Olympus DP50) using the cellSens imaging software from Olympus.

7.2.9 Phagocytosis assay on the head-kidney and posterior intestine adherent cells

A phagocytic assay was conducted to evaluate the phagocytic capabilities of the adherent cells, following the approach outlined by Carneiro et al. (2014) with adaptations. Phagocytic activity was determined using *Candida albicans* (ATCC MYA-2876), previously fixed and stained with 1 μ M Sytox Green, following established procedures. The adherent cells were cultured overnight on a cell culture dish (60 x 15 mm; Nunc™ EasYDish™, Thermo Scientific™) with a sterile glass coverslip. The non-adherent cells were eliminated by washing with warm PBS, as previously described. Subsequently, a suspension of labelled microorganism, at a ratio of 1:5, was added to the dish and the adherent cells were incubated for 20 minutes at RT. Following the 20-minute incubation, the culture dish was promptly chilled on ice to halt phagocytosis. The culture dish was then rinsed with warmed PBS to remove unbound *C. albicans*. The adherent cells were stained with propidium iodide (marker for membrane integrity) at a concentration of 6 μ L/mL for 5 minutes at RT. Lastly, fluorescence microscopy (Nikon Eclipse E400, USA) was employed to observe the cells.

7.3 Results and Discussion

In the field of aquaculture research, *in vitro* tools have emerged as invaluable assets, offering a means to investigate fundamental mechanisms ranging from immunology to immunonutrition. As the European Union's regulatory framework for animal experimentation continues to evolve, with a focus on tightening restrictions, these *in vitro* methods not only allow for a deeper comprehension of fish physiology but also play a pivotal role in reducing the reliance on live animals in compliance with increasingly stringent guidelines.

In our previous studies examining the potential of algae to modulate European seabass growth performance (Mota et al., 2023), intestinal health and microbiota (Ferreira et al., 2022; Ferreira et al., 2023c), as well as immune responses and disease resistance (Ferreira et al., 2023b), we observed that a blend of micro- and macroalgae led to improved growth, beneficial alterations in intestinal morphology, and enhanced resistance to pathogenic bacteria. Nevertheless, the underlying mechanisms responsible for these effects, particularly in relation to the immunomodulatory aspects induced by the algae, require further elucidation. Therefore, *in vitro* tools may offer a promising avenue for gaining deeper

insights into these mechanisms (Goswami et al., 2022). However, the distinct challenge in fish research is the limited availability of antibodies and established cell lines for most species, setting it apart from studies with higher vertebrates. This scarcity poses a significant barrier to advancing our understanding of fish immunology and immunonutrition, underscoring the ongoing need to develop these essential research tools tailored to the specific demands of the aquaculture field. Therefore, in the current study, our primary focus was to optimize the protocol for isolating and culturing leucocytes from the head-kidney and posterior intestine of European seabass. Given that our previous *in vivo* studies primarily emphasized the ability of algae to enhance fish resistance against bacterial diseases (Ferreira et al., 2023b), this study is specifically aimed at obtaining an enriched population of a particular leucocyte type, namely macrophages, owing to their crucial role in detecting and combating invading bacteria (Mokhtar et al., 2023).

This optimization encompassed various steps and methods, which are outlined in Table 1. Prior to the Percoll gradient separation, morphological analysis of the cells indicated the presence of erythrocytes – elongated cells with a pink cytoplasm and centrally located blue-stained nucleus – in both samples (Figure 1A). The introduction of a two-step Percoll separation, especially in the case of the posterior intestine, resulted in a reduced number of isolated cells without achieving the desired enrichment of leucocytes. Notably, the 60%/40% continuous Percoll gradient exhibited limited efficacy in separating erythrocytes. As depicted in Figure 1B, post-density separation revealed a decreased presence of erythrocytes in both samples and an enrichment of various leucocyte types: lymphocytes (small, round cells with large nuclei), monocytes/macrophages (cells with high cytoplasmic-to-nuclear ratios and vacuolated cytoplasm), and neutrophils (cells with a clear cytoplasm with segmented nuclei) (Lugo-Villarino et al., 2010). Additionally, in the intestine sample, there appeared to be a potential presence of other cell types like goblet cells (large, round cells mainly filled with mucus vacuoles), previously noted in gilthead seabream (Salinas et al., 2007). The number of cells isolated per fish ranged from $(41.0 \pm 37.7) \times 10^6$ for the head-kidney and $(5.1 \pm 3.5) \times 10^6$ for the posterior intestine, respectively. Furthermore, the viability consistently measured at $98 \pm 2\%$ for the head-kidney and $90 \pm 5\%$ for the posterior intestine. While the cell count from the European seabass intestine is lower than that reported by Salinas et al. (2007) for gilthead seabream, the viability is higher.

In summary, for the isolation of immune cells from the head-kidney and intestine of European seabass, the recommended procedure that yields the highest number of viable cells includes mechanical dissociation for the head-kidney and, for the intestine, a prior treatment with DTT for 15 minutes followed by 2 hours of collagenase treatment with agitation before mechanical dissociation. Subsequently, a single step of Percoll separation, to isolate immune cells from other cellular components, is to be employed, utilizing the

following gradients: 34% and 51% for the head-kidney and 25% and 75% for the intestine. Overall, the head-kidney leucocyte isolation method produced enough viable cells for future experiments, but improvements are required for isolating leucocytes from the posterior intestine.

Table 1. Steps and methods tested during the protocol optimization for cell isolation and detachment.

Isolation				
Head-kidney	Method for leucocyte isolation	Hypotonic lysis of erythrocytes		
		Percoll Density Gradient Centrifugation	40%/60% continuous Percoll gradient	Single step separation
			34%/51% continuous Percoll gradient	Single or double step separation
Posterior intestine	Tissue dissociation	Time of incubation (30 min or 2 hours)		
		With and without agitation		
	Method for leucocyte isolation	Percoll Density Gradient Centrifugation	25%/75% continuous Percoll gradient	Single-step separation
				Double step separation
Detachment				
Detachment solution	PBS 1x, supplemented with 5 mM EDTA			
	Macrophage detachment solution			

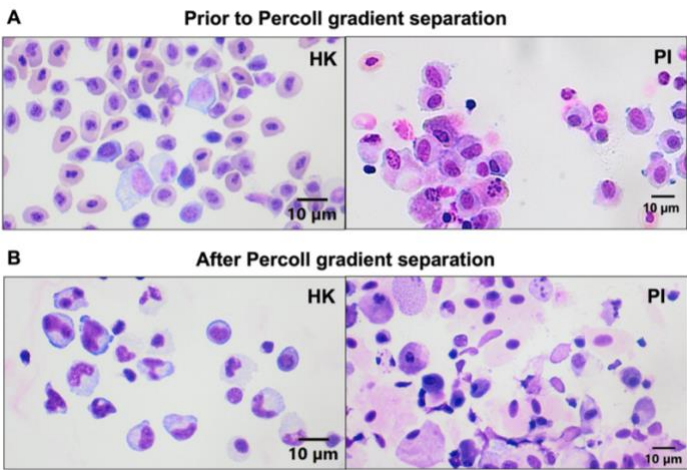


Figure 1. Representative images of the isolated cells from the head-kidney and posterior intestine prior to Percoll gradient separation (A) and following density separation (B).

To potentially distinguish different immune cell populations within the head-kidney and posterior intestine, FACS was employed, utilizing WGA and LEL lectins for

immunophenotyping. Typically, samples are labelled with fluorescent proteins or fluorophore-conjugated antibodies to define specific cell populations through flow cytometry or fluorescence microscopy. However, in the context of fish species, particularly the European seabass, a limitation arises from the absence of antibodies specific to lineage markers (Fei et al., 2021). Consequently, lectins serve as a valuable alternative, binding to characteristic glycans on live cell surfaces. As shown in Figure 2, lectin binding affinity guided the isolation of various cell populations. In the head-kidney samples, five subpopulations were identified – LEL⁺WGA⁺; LEL^{int}; LEL⁺WGA^{int}; WGA^{int}; LEL⁺WGA⁻ (Figure 2A), and in the posterior intestine samples, three subpopulations were sorted – LEL⁺; WGA⁺; LEL⁻WGA⁻ (Figure 2B). The sorted cell populations were found to consist of mixed cell types (e.g., lymphocytes, monocytes, erythrocytes) based on morphological analysis. Therefore, it appears that the observed differences in marker expression levels can be attributed to individual variations in isolated cells rather than being associated with specific cell types. Lectins have been successfully applied to differentiate between immune cell types in Pacific oysters (*Crassostrea gigas*) (Jiang et al., 2016) and whiteleg shrimp (*Litopenaeus vannamei*) (Estrada et al., 2016) but in the present study immune cell subtypes could not be classified through LEL and WGA binding in European seabass.

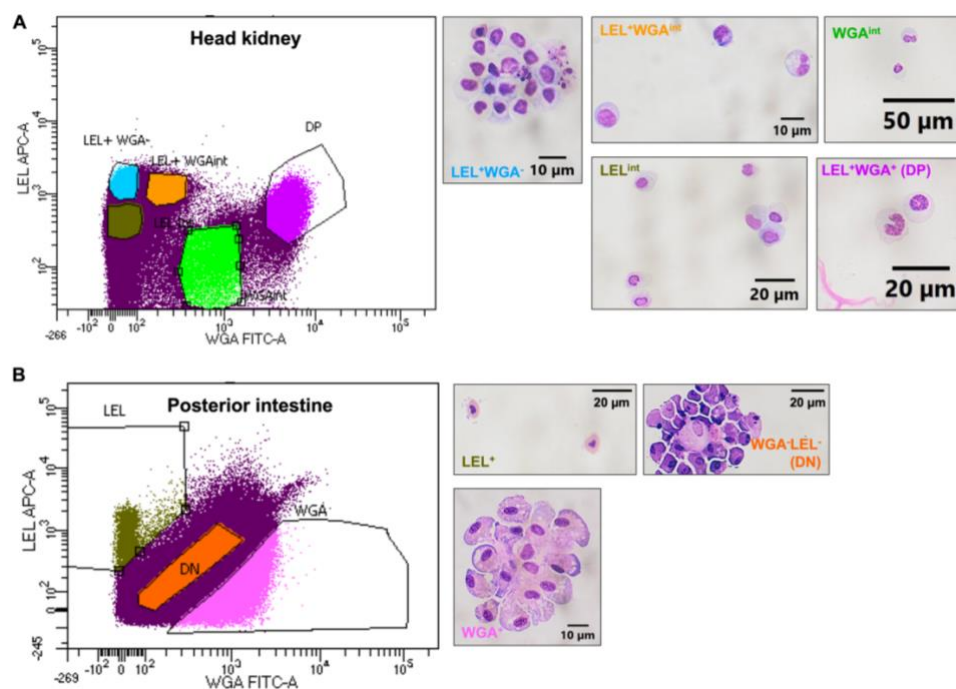


Figure 2. Characterization of the subpopulations of cells identified in head-kidney (A) and posterior intestine (B). The subpopulations were sorted by FACS, through differential lectin-binding affinity (WGA and LEL).

To obtain subfractions enriched in specific cell types, methods other than immunophenotyping have been employed. Studies conducted on the head-kidney of various fish species, including rainbow trout (MacKenzie et al., 2003), Atlantic salmon (Park et al., 2020a), Persian sturgeon (*Acipenser persicus*) and Caspian kutum (*Rutilus kutum*) (Rastgar et al., 2018), and European seabass (Sarmiento et al., 2004), have established monolayer cultures and reported an enrichment of monocyte/macrophage type cells within the subculture. Additionally, a similar approach was employed by Park et al. (2020b) to isolate an enriched population of macrophage-like cells from the distal intestine of Atlantic salmon. To confirm the attachment ability of the isolated cells and assess the morphological characteristics of these cells, the isolated cells were cultured overnight on a coverslip after a thorough rinse with warm media to remove all non-adherent cells. After cell isolation, approximately 2.5×10^6 head-kidney cells were plated, resulting in an average of 1.15×10^6 cells post-detachment. Similarly, after culturing around 2.5×10^6 posterior intestine cells, an average of 4.2×10^4 adherent cells were detached. This difference in recovered adherent cells between the head-kidney and intestine is anticipated due to the expected higher count of immune cells, particularly macrophages, in the head-kidney (haematopoietic organ) compared to the intestine in healthy fish. The yield of harvested cells was similar when contrasting the results from the use of PBS with EDTA 5 mM or the macrophage detachment solution. As can be observed in Figure 3, the cells projected onto the surface of the plate, a behaviour characteristic of adherent cells. It was hypothesized that the adherent cell population, distinguished by their morphology characterized by high cytoplasmic-to-nuclear ratios, primarily comprised monocytes and macrophages. However, it's important to note that other immune cells, such as neutrophils and dendritic cells, also possess adherent capacity and could potentially be part of this population (Lugo-Villarino et al., 2010). Therefore, further analysis of the expression patterns of these cells is necessary to gain additional insights into the specific cell types present within the adherent cell population.

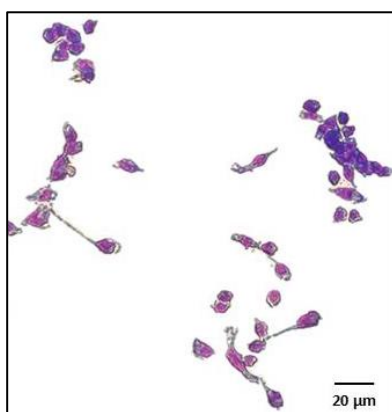


Figure 3. Morphological characterization of the adherent cells attached a cover slip and observed under an inverted optical microscope.

Furthermore, functional assays were conducted to evaluate the phagocytic capacity of the adherent cells. As depicted in Figure 4, the internalization of Sytox Green-labelled *C. albicans* by the head-kidney adherent cells of European seabass suggested their phagocytic activity. The intestinal adherent cells exhibited similar behaviour. Given that *C. albicans* (5-6 μm in diameter) is relatively large compared to common fish pathogenic bacteria like *Vibrio anguillarum* (0.5-1 μm), it is hypothesized that these adherent cells would likely exhibit phagocytic activity against pathogenic bacteria relevant within the context of aquaculture. This observation is consistent with the findings of Park et al. (2020b), where adherent cells from the head-kidney and posterior intestine of Atlantic salmon exhibited phagocytic activity against *Escherichia coli* BioParticles and expressed genes associated with macrophages. It is, however, important to note that besides macrophages, neutrophils, and dendritic cells are also recognized as professional phagocytes (Silva & Correia-Neves, 2012).

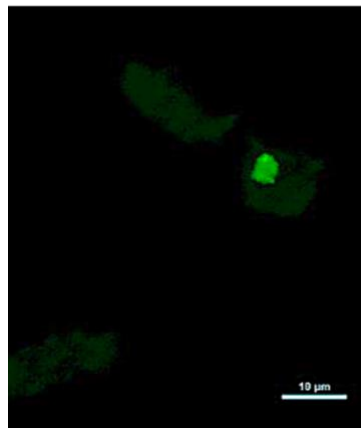


Figure 4. Head-kidney adherent cells with internalized Sytox Green-labelled *Candida albicans*.

7.4 Conclusion

In this study, we established protocols for isolating and culturing leucocytes from two vital organs involved in the systemic and mucosal immunity of European seabass: the head-kidney and the intestine. The leucocyte isolation protocol for the head-kidney successfully generated a sufficient quantity of viable cells, which can be utilized in future *in vitro* assays for assessing novel functional compounds. However, further refinements are needed for the protocol related to isolating leucocytes from the posterior intestine.

In the effort to isolate specific subpopulations of leucocytes, the selective binding of lectins WGA and LEL to cells was evaluated. Although variations in lectin binding affinity were observed, the characterization of the sorted subpopulations confirmed that these populations were not selectively enriched in particular leucocyte types. Nevertheless, morphological and functional characteristics similar to those of macrophage-like cells, which

were the primary focus of this study, were exhibited by adherent cells from the head-kidney and posterior intestine of European seabass. Before future experiments, a comprehensive characterization of these cells, such as through transcriptomics or single cell sequencing analysis, is imperative to substantiate this hypothesis.

Our study represents a crucial initial step in the development of *in vitro* tools aimed at understanding the immune system of the European seabass and assessing the role of immunonutrition in fish health. Additionally, these innovative approaches contribute to the progress of more ethical and sustainable practices in the field of aquaculture.

7.5 Author contributions

Mariana Ferreira: conceptualization, methodology, formal analysis, investigation, data curation, and writing – original draft preparation. Daniela Salazar: formal analysis, investigation, data curation, and writing – original draft preparation. Youngjin Park: conceptualization, methodology, investigation, and writing – review and editing. Alexandra Correia: methodology and writing – review and editing; Manuel Vilanova – methodology and writing – review and editing; Luisa M. P. Valente: funding acquisition, validation, supervision and writing – review and editing. Kiron Viswanath: methodology, validation, supervision and writing – review and editing. Sónia Gomes: funding acquisition, conceptualization, methodology, investigation, and writing – review and editing.

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7.7 Acknowledgements

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Chapter 8.

General discussion, conclusions, and future approaches

Boosting fish robustness: Unveiling the potential of algae feeds for enhanced intestinal health and performance

OBJECTIVES:

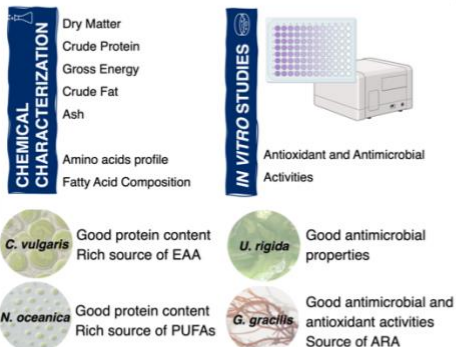
Understand the potential of algae to improve farmed fish robustness through the dietary modulating of intestinal health
Provide improved and/or new methods for the evaluation of fish intestinal health

Chapter 2

Selection of commercially available algae for *in vivo* trials, based on their nutritional value and bioactivity



Micro- and Macroalgae Blend: ALGAESSENCE™ - Feed



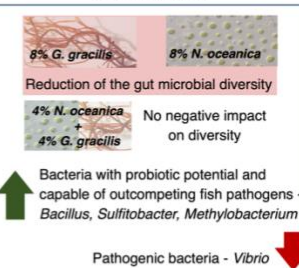
ALGAESSENCE™ - Feed: well-balanced nutritional composition and broad-spectrum effect, with highest antimicrobial activity against *Tenacibaculum maritimum*

Chapter 3

Understand the impact of algae on the intestinal histomorphology and microbiota

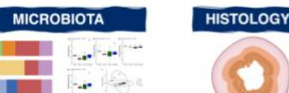


Feeding Trial 1

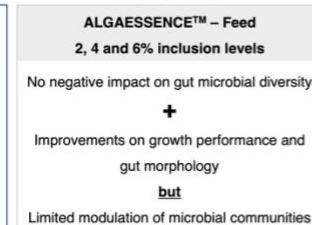


Chapter 4

Understand the impact of algae on the intestinal histomorphology and microbiota



Feeding Trial 2

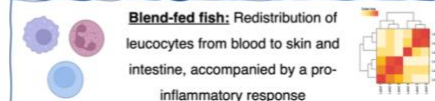


Chapter 5

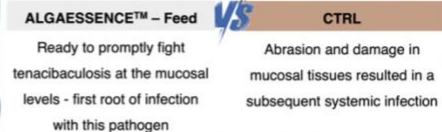
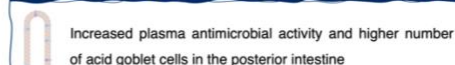
Unravel the immunomodulatory potential of the algae blend (ALGAESSENCE™ - Feed)

Following Feeding Trial 2 - Bacterial BATH Challenge
Tenacibaculum maritimum

HEMATOLOGICAL PROFILE AND GENE EXPRESSION

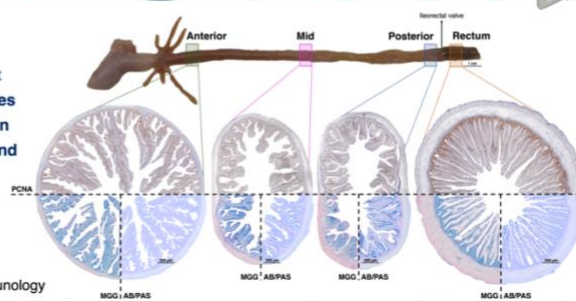


PLASMA INNATE PARAMETERS AND GUT HISTOLOGY



Chapter 6

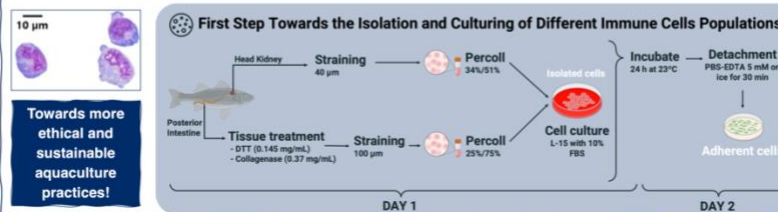
Select the right segment and morphometric indices for future experiments on nutritional modulation and intestinal health



Anterior: Nutrition trials
Posterior and Rectum: Immunology

Chapter 7

Development of *in vitro* tools to test novel functional compounds



8.1 General discussion

Over recent years, aquaculture has played a crucial role in global food production, addressing the growing demand for protein (Boyd et al., 2022). Yet, the aquaculture sector faces complex challenges, particularly due to the increasing incidence of disease outbreaks in fish farms resulting from the intensification of farming practices and the difficulties arising from climate change. To tackle these challenges necessitates a multifaceted approach that integrates the use of innovative dietary strategies with the examination of fish microbiota, immune responses, and the development of advanced tools for assessing fish intestinal health (Boyd et al., 2020; Cascarano et al., 2021; Dawood, 2021).

8.1.1 Micro- and macroalgae as promising dietary components for aquafeeds

The composition of aquafeeds plays a pivotal role in shaping the health and disease resistance of farmed fish (Dawood, 2021). Notably, micro- and macroalgae have emerged as promising candidates for enhancing the nutritional content of fish diets and boosting their immune systems (Valente et al., 2021; Wan et al., 2019). In Chapter 2, a comprehensive examination of various commercially available algae species highlights their potential as dietary components for aquafeeds. This research emphasises the significance of algae's protein content, their profiles of EAA and fatty acids, and their ability to address imbalances associated with PP sources.

Indeed, the feed industry is continually on the lookout for alternative protein sources, primarily driven by a confluence of social, economic, and environmental factors (Yue & Shen, 2022). Conventional protein sources like FM and soybean are not only resource-intensive but are also susceptible to price volatility due to market forces and climate-related disruptions (Gephart et al., 2020). In response to these challenges, microalgae offer a compelling solution due to their high contents in protein but also reduced resource requirements compared to traditional crops (Shah et al., 2018). Furthermore, algae, such as *Chlorella* sp., naturally possess a wide range of EAA, often in concentrations that are comparable to or even exceeding those found in traditional animal-based protein sources. This intrinsic amino acid profile in algae, including a significant content of lysine, a limiting EAA in the most frequently used vegetable ingredients in feed formulations (Leinonen et al., 2019), offers a valuable alternative for formulating aquafeeds. Additionally, algae like *Nannochloropsis* sp., besides being rich protein sources, serve as natural producers of PUFAs. Many marine fish species, such as European seabass, heavily rely on external sources of PUFAs, particularly EPA and DHA, for their growth, health, and overall development (Tocher, 2010). Alongside *N. oceanica*, macroalgae *G. gracilis* is a rich source

of ARA, an essential PUFA for marine fish. Thus, incorporating algae species, which PUFAs closely mirror the natural sources of omega-3 fatty acids found in marine ecosystems, can be a highly effective strategy for enhancing the nutritional content of aquafeeds for farmed fish species. Moreover, macroalgae such as *G. gracilis* and *U. rigida* contain high carbohydrate content, ranging from 46% to 53%. Interestingly, macroalgal polysaccharides have known prebiotic, antimicrobial, and antioxidant properties that could potentially offer health benefits for farmed animals (Wijesekara et al., 2011).

Chapter 2 also delves into the antimicrobial and antioxidant potential of these algae. The findings reveal their antibacterial activity against various pathogens, holding significant implications for managing bacterial infections in aquaculture. Both micro- and macroalgae display antimicrobial activities, with the blend showing promise in achieving a broad-spectrum effect. Indeed, the antimicrobial activity of the tested algae against *T. maritimum*, an important fish pathogen, highlights the potential of nutritional strategies, such as algae, to control disease outbreaks in aquaculture.

This research highlights the advantages of algae, pointing towards their inclusion in aquafeeds as functional additives that can enhance fish growth and health while aligning with sustainability principles. However, it also reveals the need for further studies to unlock the full potential of these algae products in both *in vitro* and *in vivo* applications within the aquaculture industry. Among the tested algae, the commercial micro- (*N. oceanica* and *C. vulgaris*) and macroalgae (*G. gracilis* and *U. rigida*) blend, ALGAESSENCE™ – Feed, stands out as a well-balanced product with broad-spectrum effects due to its richness in proteins, lipids, EAA, essential fatty acids (EPA and ARA), and carbohydrates. Use of algae blends is a pioneering approach by valorising the bioactive potential of more expensive algae, such as *Nannochloropsis* sp., by seamlessly integrating different algae in a single product. This innovative approach leverages the strengths of each alga, capitalizing on their high protein, amino acids and fatty acid contents to create a holistic and sustainable solution that redefines the possibilities of algae-based products in the industry.

8.1.2 Impact of algae on the intestinal microbiota and morphology of European seabass

Chapter 3 and 4 delve into the impact of these algae on fish microbiota, recognizing its critical role in fish health and disease resistance (Dawood, 2021; López Nadal et al., 2020). In Chapter 3, the focus is on the effects of including macroalgae (*G. gracilis*) and microalgae (*N. oceanica*), both separately and in a blend, in the diets of European seabass at an inclusion level of 8%. This research identifies a core microbiome in European seabass consisting of potentially beneficial bacteria, such as *Lactobacillus* and *Cetobacterium*, which

exhibit close phylogenetic relationships with species known for their probiotic activities *in vitro* and *in vivo*, like *Lactobacillus delbrueckii* subsp. *lactis*, *Lactobacillus acidophilus*, *Lactobacillus helveticus*, and *Lactiplantibacillus plantarum* (Ahire et al., 2019; Hosseini et al., 2016; Iorizzo et al., 2022; Rurangwa et al., 2009) and *Cetobacterium somerae* (Xie et al., 2022; Xie et al., 2021b; Zhou et al., 2022). Additionally, an identified *Flavobacterium*, exhibiting high similarity to a potentially pathogenic bacterial species, *Flavobacterium succinicans* (Good et al., 2015), was part of this core microbiota. As constituents of the core microbiota, these bacteria likely play a pivotal role in maintaining biological functions in European seabass. Consequently, the presence of both beneficial and potentially pathogenic bacteria within this core emphasizes the significance of sustaining a balanced microbial community for the health and disease resistance of the host.

Moreover, the research reveals significant changes in the gut microbiota of European seabass when incorporating these algae into their diet over a 3-month feeding trial, affecting the abundance of various bacterial groups in the fish intestine. While bacterial diversity decreased with single algae inclusion, the blend appeared to mitigate some of these effects. It is important to note that a decrease in bacterial diversity does not always indicate a negative impact (Gupta et al., 2019); it is crucial to investigate the specific types of bacteria being affected. For instance, the *G. gracilis* diet resulted in a decrease in the abundance of bacteria involved in nutrient degradation and utilization (i.e., *Opitutus* and *Rhizobium*), potentially negatively impacting digestion and nutrient absorption processes. In our related study by Batista et al. (2020a), we observed decreased protein and energy digestibility in fish on the macroalgae-supplemented diet, yet without impacting growth performance. However, diets rich in micro- and macroalgae promoted the growth of bacteria possibly possessing probiotic properties, such as *Sulfitobacter*, *Methylobacterium* and *Bacillus* (Boutin et al., 2013; Wilczynski et al., 2022). Specifically, the *Bacillus* ASV modulated in our study was closely related to strains exhibiting antimicrobial and probiotic activities like *Geobacillus thermoleovorans*, *Caldibacillus thermoamylovorans* and *Caldibacillus hisashii* that exhibit antimicrobial and probiotic activities (Inabu et al., 2022; Zebrowska et al., 2022). Conversely, the inclusion of *N. oceanica* also reduced potentially pathogenic bacteria like *Acinetobacter* and *Vibrio*, closely related to species reported as fish pathogens such as *Acinetobacter pittii* (Cao et al., 2018; Malick et al., 2020) and *Vibrio fortis* (Austin et al., 2005). These findings indicate that algae have the potential to modulate various bacterial groups in the intestine of European seabass, with implications for host health, nutrition, and disease resistance.

The previous chapters illustrate the added-value potential of algae blends, compared to single micro- and macroalgae, both in terms of chemical composition, bioactivity, and microbiota modulation. Therefore, in Chapter 4, we delve into the potential of the

abovementioned ALGAESSENCE™ – Feed blend, composed of two microalgae and two macroalgae, to modulate the intestinal microbiota of European seabass. The algae blend was tested at three different incorporation levels (2%, 4%, and 6%) to determine the optimal inclusion level for promoting functionality. The inclusion of ALGAESSENCE™ – Feed led to observable changes in gut structure, including increased villus height, muscularis thickness, and a higher number of acid goblet cells. These changes were accompanied by improved growth rates, with more pronounced effects seen at the higher inclusion levels, particularly at 4% and 6%. These results suggest a positive influence of the algae blend on factors like absorption area, intestinal motility, and mucosal immunity (Dawood, 2021; Kim & Khan, 2013; Olsson & Holmgren, 2001).

However, there were no significant changes in intestinal microbial diversity in fish consuming the blend diet. Furthermore, the modulation of bacterial groups within the gut was confined to fish fed the 2% blend diet when compared to all other tested diets (CTRL, Blend 4 and Blend 6). This modulation affected both bacteria with pathogenic potential and probiotic properties, with no clear positive or negative trend. These modest effects on the gut microbiota imply that while the algae blend appears to have an overall positive impact on gut health, other physiological factors may be responsible for the observed phenotypical changes, such as improved growth performance and alterations in intestinal structure.

Collectively, Chapters 3 and 4 establish a foundational understanding of the complex interplay between dietary components and the gut microbiota. In Chapter 3, higher incorporations (8%) resulted in more pronounced differences in both the diversity and composition of the gut microbiota. Although both micro- and macroalgae positively influenced bacteria with potential probiotic activity, microalgae appeared to have a more favorable impact on the gut microbiota by increasing beneficial bacteria and decreasing potentially pathogenic ones. While a previous complementary study by Batista et al. (2020a) showed that *G. gracilis* negatively affected nutrient digestibility, this was not observed in fish fed the microalgae-supplemented diets. However, no significant differences in growth performance were noted, and effects on intestinal structure were limited compared to fish on the control diet (Batista et al., 2020a).

In contrast, lower incorporations (2%, 4%, and 6%) of an algal blend in Chapter 4 demonstrated positive effects on growth and gut structure but had minimal impact on the gut microbiota. Thus, the potential positive effects observed in fish with the tested levels of commercial algae blend incorporation did not seem to correspond with changes in the intestinal microbiota. Additionally, although all tested diets remained isonitrogenous, it is worth noting that in Chapter 3, the algae were included at the expense of FM and wheat meal in diets, whereas in Chapter 4, the algae blend exclusively substituted vegetable sources (whole peas and wheat gluten). The observed positive effects on intestinal structure

and growth performance in fish fed the commercial algae blend suggest that algae could be a promising alternative ingredient to vegetable protein sources commonly used in today's formulations. In the current socio-economic context, vegetable products are becoming scarcer and more expensive (Ben Hassen & El Bilali, 2022). Despite algae still being relatively costly, they might present a viable alternative in the near future. Increased interest from the food and feed market is expected to contribute to price reduction, as economies of scale begin to take effect (Diaz et al., 2023; Thornton et al., 2023).

Overall, algae can have a positive impact on the gut microbiota composition and can be used to formulate more effective and health-promoting aquafeeds. For instance, the role of dietary components in promoting the growth of beneficial bacteria, which can outcompete pathogenic ones, is crucial for disease resistance in aquaculture. By creating an environment within the fish that is less conducive to the growth of harmful microorganisms, the likelihood of disease outbreaks in fish farms is reduced. This, in turn, minimizes the need for antibiotics and other disease management interventions, contributing to more sustainable and environmentally friendly aquaculture practices (Dawood, 2021; Hoseinifar et al., 2019). However, the depth of the utilized methodology, such as 16s rRNA sequencing, limits the assessment of microbial population changes as it does not reach the species level. To address this limitation, our study conducted a phylogenetic analysis, comparing altered ASVs with the NCBI database, offering insights into potential functional diversity among bacterial groups influenced by the algae diets. Nevertheless, future investigations should employ advanced methods to precisely identify the specific bacterial species affected by the diets and their functional implications. Furthermore, more studies are necessary to comprehend the individual impacts of each algae species on gut microbiota, as well as to determine the most effective inclusion levels and formulation strategies (e.g., replacing FM or vegetable products) to maximize algae's potential as aquafeed ingredients.

8.1.3 Potential of algae modulate the immune responses of European seabass

With the foundation laid in Chapters 3 and 4, Chapter 5 further investigates the impact of the algae blend (ALGAESSENCE™ – Feed) on fish health, by focusing on the immune system and overall fish robustness. The primary emphasis lies in understanding its immunomodulatory capacity and how it influences the fish's ability to fight the pathogen *T. maritimum*, which is responsible for ulcerative disease in European seabass. After feeding seabass juveniles a diet containing this algae blend for 12 weeks and subsequently challenging them with *T. maritimum*, the study reveals noteworthy findings. The algae blend

seems to promote an adequate mucosal immune response upon infection with *T. maritimum*, enhancing resistance to the pathogen.

This immunomodulation encompasses systemic and mucosal responses, offering protection against infections, including those of the pathogen *T. maritimum*. At the systemic level, the increased plasma antimicrobial activity observed with algae blend inclusion does not align with the reduction in lysozyme activity seen in Blend 6 fish. These results suggest that the algae may influence other immune components, such as antimicrobial peptides like hepcidin, which showed elevated gene expression levels in the head-kidney, potentially contributing to the increased antimicrobial activity in the plasma. Regarding mucosal responses, two primary organs were evaluated: the skin and the intestine. As a bath challenge was employed to simulate the route of infection with this bacterium, the anticipated modulation of immune parameters at the skin level, enhanced by the algae diets, was expected since the skin is the primary site affected by this pathogen (Ferreira et al., 2023a). Notably, observed alterations in immune gene expression and the increased number of acid goblet cells in the intestine, further stimulated by the algae blend, emphasize the significance of intestinal health in modulating resistance against this pathogen.

When considering the results from Chapter 4, where the algae blend had a limited impact on the gut microbiota of European seabass, it appears inconsistent with the pronounced modulation of the immune response observed in European seabass upon *T. maritimum* infection. However, it is important to note that the microbiota analysis was conducted after the feeding trial. Performing the same analysis after the subsequent infection trial could provide insights into whether the microbiota responds differently to the diet under challenging conditions. Conversely, the favourable effects observed on growth performance and gut structure, detailed in Chapter 4 – specifically the increased villus height and greater number of acid goblet cells, known for their protective role against bacterial translocation – could potentially have synergistically enhanced the seabass' response against this pathogen. Furthermore, the *in vitro* antimicrobial activity of the algae blend against *T. maritimum* observed in Chapter 2 provides further support to these results.

In exploring the significance of feed formulation and the incorporation levels of potentially functional ingredients like algae, three different incorporation levels (2%, 4%, and 6%) were examined. The study suggests that a 4% incorporation level of the algae blend might be optimal, within the tested range, for enhancing the resistance of European seabass to *T. maritimum* infection. However, it underscores the necessity for further research to fine-tune the inclusion levels of this algae blend in aquafeeds. Despite Blend 4 showing better immune modulation in most parameters and lower mortality, Blend 6 displayed superior results in terms of plasma antimicrobial activity and growth performance. Therefore, refining the doses, particularly between 4% and 6%, is crucial to identify the most cost-effective

solution. Considering that algae remain relatively expensive resources, determining the lowest incorporation level that can achieve the highest growth and yield the most robust fish becomes a primary goal for fish producers.

To sum up, Chapter 5 highlights the potential of this commercial algae blend as a dietary supplement to boost the immune responses of farmed European seabass. These findings are particularly relevant in the current context, as tenacibaculosis, originally reported in Japan, has been spreading throughout Europe, impacting the production of various farmed fish species such as the European seabass (Ferreira et al., 2023a). Additionally, the absence of a commercially available vaccine specifically targeted for seabass against *T. maritimum* emphasizes the critical need for innovative strategies to enhance the resilience of the fish (Mabrok et al., 2022). Algae-based immunonutrition could prove particularly beneficial for fish larval stages that lack a fully developed immune system and can undergo vaccination only in later stage of its life (Buchmann, 2022). Overall, the inclusion of algae blends in aquafeeds holds the potential to benefit not only European seabass but also other farmed fish species and various developmental stages, ultimately enhancing their immune status and disease resistance.

8.1.4 Detailed characterization of the European seabass intestine

The complex evaluation of fish intestinal health relies on standardized procedures and meticulous descriptions of the gastrointestinal tract. Unfortunately, comprehensive, and accurate descriptions of the morphology of the gastrointestinal tract of farmed fish are limited (Bjørngen et al., 2020; Verdile et al., 2020b). Moreover, many studies fail to provide a precise description of the intestinal portions used in their analysis, and the lack of standardized terminology adds to the confusion. This makes it challenging to compare results with existing literature (Vatsos, 2021). In response to these challenges, in Chapter 6, a detailed characterization of the European seabass intestine was conducted, categorizing it into four distinct segments: anterior, mid, posterior, and rectum. Each segment exhibits unique histomorphological and gene expression profiles. The anterior intestine is associated with digestion and absorption processes, featuring a high absorptive area, long villi, and expressions of genes related to carbohydrate and lipid digestion. In contrast, the rectum and posterior intestine show immune-related characteristics, with high expression of immune-related genes, a higher number of acid goblet cells (involved in immune defence), and a thick muscularis in the rectum, suggesting its role in waste release. The mid intestine serves as an intermediate region for both absorption and immune processes.

Furthermore, this chapter introduces a semi-quantitative scoring system and a quantitative approach for measuring various parameters in the intestine, providing researchers with reliable methods to assess the impact of diets and feed components on intestinal health. The findings offer valuable insights into seabass intestinal morphology and underline the importance of precise and standardized methodologies in research concerning the impact of diets on intestinal functionality. The methodologies provided in this chapter can serve as a valuable reference for future research in the field of aquaculture. It can be used as a basis for comparing results across different studies and ensuring that research in this area is consistent and reliable. The ultimate goal is to incorporate digital technologies and artificial intelligence for high-throughput image analysis, streamlining the evaluation process. This technological advancement would significantly reduce the time and effort typically associated with histological evaluations of intestinal health, which are often laborious and time-consuming. Simultaneously, it would produce robust and fast-tracking data (Jing et al., 2023).

Indeed, the standardized methods outlined in Chapter 6 were put into practice to investigate the impact of a macro- and microalgae blend, composed of *Gracilaria* sp., *Nannochloropsis* sp. and *Aurantiochytrium* sp., on European seabass intestinal health. Although the dietary inclusion of the algae blend did not notably influence the fish's growth performance or feed utilization parameters throughout the 8-week feeding trial, it did reveal potential immunomodulatory effects. This aligns with findings from Chapter 5, highlighting the algae's ability to induce an immunomodulatory effect and showcasing its potential in fortifying fish immunity and resilience.

Of particular interest were the significant responses observed in the mid intestine, suggesting its susceptibility to external stimuli. While both histomorphological and gene expression patterns were influenced by the algae blend in the mid portion, variations in the anterior, posterior and rectal sections were primarily at the gene expression level. However, comparing studies targeting the intestine poses challenges due to its heterogeneity, as different sections may respond differently to a diet. The mid portion, located between the anterior and posterior intestine, lacks clear physical markers. Conversely, identifying the anterior, posterior, and rectal sections based on specific landmarks – the anterior following the pyloric caeca, the posterior preceding the ileorectal valve, and the rectum after this valve – facilitates more precise sampling. Hence, studies involving these regions can be more easily compared than those focusing on the mid section.

To sum up, characterization of the different segments of the gastrointestinal tract is essential for understanding how European seabass responds to different dietary treatments. This comprehensive characterization allows us to gain deeper insights into the specific functions and responses of each intestinal segment to dietary interventions. Our

research highlights that the anterior portion is the most relevant segment for studies targeting digestive processes. On the other hand, the rectum and posterior intestine may hold greater relevance for studies focusing on immunonutrition. This knowledge is instrumental in optimizing aquafeed formulations to enhance nutrient absorption, immunonutrition, and overall growth in farmed fish (Dawood, 2021).

8.1.5 Optimization of protocols for the isolation of immune cells from the head-kidney and intestine of European seabass

As the scientific community continues to strengthen regulations regarding animal experimentation, there is a growing necessity to create *in vitro* tools capable of replicating the *in vivo* environment (Ferdowsian & Beck, 2011). In the realm of aquaculture research, particularly within the scope of immunonutrition, examining immune cells from the fish intestine could offer insights into understanding how functional products, such as algae, impact mucosal immunity. However, as established commercial fish cell lines are limited in availability, it became imperative to develop and optimize protocols for isolating and culturing primary immune cells from both the head-kidney and intestine of European seabass. This focus on developing protocols is the essence of Chapter 7.

The optimised protocol yielded a substantial number of viable leucocytes from the head-kidney, suitable for future *in vitro* assays. Conversely, the isolation protocol for posterior intestine leucocytes requires further refinement. Since our focus is on algae from an immunonutrition standpoint, considering the rise in bacterial outbreaks observed in farms, we concentrated on macrophages, which play a central role in defending the host from infection (Mokhtar et al., 2023). However, specific antibodies for sorting immune cell populations from European seabass are currently unavailable. We explored alternative approaches, such as examining the expression patterns of lectins wheat germ agglutinin (WGA) and *Lycopersicon esculentum* (tomato) lectin (LEL) to distinguish specific leucocyte subpopulations. While we observed differences in lectin binding affinity, the subsequent characterization of sorted subpopulations revealed that they were not selectively enriched in particular leucocyte types.

In this study, we employed various strategies to obtain an enriched population of the target cells – macrophages. Leveraging the known adherent capacity and phagocytosis ability of macrophages, we successfully enriched this cell population by isolating, culturing, and recovering attached cells. Based on the observed morphology of the adherent cells, it was theorized that the recovered cell population was enriched in macrophages. However, it is crucial to note that both neutrophils and dendritic cells also exhibit adherent capacity and phagocytosis ability, raising the possibility of their presence within the adherent cell

population. Therefore, it is imperative to conduct further characterization of the obtained cells to validate and substantiate these preliminary findings.

Overall, this chapter provides crucial insights into the development of *in vitro* tools for a deeper understanding of the European seabass's immune system. These tools will allow for a more profound exploration of the cellular and molecular processes behind the observed *in vivo* effects of the algae discussed in previous chapters, shedding light on the intricate web of interactions between diet, microbiota, and the immune system. Such knowledge can be instrumental in designing aquafeeds that enhance health and disease resistance in farmed fish. Furthermore, these *in vitro* tools can also significantly reduce the need for *in vivo* experiments, which can be more resource-intensive and ethically challenging (Goswami et al., 2022).

8.2 Conclusions

This comprehensive research project presents a holistic approach that addresses crucial challenges in aquaculture, presenting key insights:

- a. **Algae's significance in aquafeeds:** Chapter 2 highlights the pivotal role of algae in aquafeed formulations. It emphasizes how micro- and macroalgae offer diverse nutritional benefits, including rich protein content, EAA, and fatty acids, contributing significantly to fish health and disease resistance. Moreover, the antimicrobial and antioxidant properties exhibited by algae underscore their potential for disease management in aquaculture.
- b. **Algae's impact on fish gut microbiota:** Chapter 3 and 4 investigations into the impact of micro- and macroalgae on European seabass gut microbiota revealed distinct alterations. Chapter 3 noted significant shifts in microbiota composition with higher algae incorporations (8%), influencing beneficial and potentially pathogenic bacteria. In contrast, lower algae incorporations (2%, 4%, and 6%) in Chapter 4 showed limited changes in microbiota composition despite positive effects on fish growth and gut structure. These varying impacts underscore the complexity of how different algae inclusion levels can selectively modulate the gut microbiota of European seabass.
- c. **Algae's role in modulating fish immune response and robustness:** Chapter 5 investigates the impact of a micro- and macroalgae blend on European seabass health, revealing an enhanced mucosal and systemic immune responses, promoted by the algae blend, upon subsequent *T. maritimum* challenge. Notably, the algae blend stimulates elevated plasma antimicrobial activity and alterations in immune gene expression in both skin and intestine, emphasizing the significance of intestinal health in resisting pathogens. Although a 4% inclusion of the algae blend seems optimal for

enhancing European seabass robustness, Blend 6 displayed superior growth performance, signalling the need for fine-tuning the inclusion levels to balance immune response, reduced mortality, and cost-effectiveness.

- d. Intestinal characterization and standardized methodologies:** Chapter 6 presents a detailed characterization of the European seabass intestine, outlining four distinct segments with unique histomorphological and gene expression profiles. The anterior intestine primarily focuses on digestion and absorption, whereas the rectum and posterior intestine exhibit immune-related characteristics. Introducing a semi-quantitative scoring system and quantitative approach aids in evaluating intestinal health, providing standardized methodologies crucial for consistent and reliable research in aquaculture. By employing the standardized methodologies outlined in Chapter 6, the investigation into the micro- and macroalgae blend's impact on seabass intestinal health showcased potential immunomodulatory effects.
- e. *In vitro* tools development for seabass immune system study:** Chapter 7 establishes protocols for isolating and culturing primary immune cells from the head-kidney and intestine of European seabass. While successful in obtaining viable leucocytes from the head-kidney, further refinement is required for isolating posterior intestine leucocytes. Strategies explored offer preliminary insights into potential cell population enrichment, particularly macrophages, providing groundwork for deeper understanding of interactions between diet, microbiota, and immunity in farmed fish.

In summary, these findings significantly advance our understanding of algae's pivotal role in sustainable aquaculture, introduce standardized methods for assessing fish intestinal health, and pave the way for future advancements in *in vitro* tools. This interdisciplinary and comprehensive project offers potential solutions benefiting researchers and fish farmers alike, showcasing the value of such collaborative efforts in addressing complex challenges in aquaculture.

8.3 Future approaches

The complex interconnection between fish, their intestinal microbiota, and their overall health and performance is a central focus of research in the field of aquaculture (Yukgehnash et al., 2020). Building on Chapters 3 and 4, focused on the investigation of the intestinal microbiota, future studies can delve deeper into the mechanisms through which algae affect the microbiome. In this context, metagenomics and metatranscriptomics offer significant advantages over the conventional 16S rRNA sequencing for microbiota analysis. These advanced techniques allow for the identification and differentiation of closely related species and strains, enabling a more comprehensive exploration of

microbiota diversity. Furthermore, they provide insights into functional profiling by revealing the genes and metabolic pathways within the microbiota and shed light on its active functions and responses under specific conditions (Bharti & Grimm, 2019). The use of multi-omics techniques – metagenomics, metatranscriptomics, metaproteomics, and metabolomics – can provide a holistic view of the complex interactions between algae, fish, and their microbiota. This integrated approach can offer a deeper understanding of the mechanisms at play (Nyholm et al., 2020).

This thesis offers valuable insights into the potential of algae as functional ingredients for aquafeeds and lays the foundation for future approaches to evaluate the role of immunonutrition in aquaculture. Algae are rich sources of bioactive compounds that can be harnessed for functional purposes (Valente et al., 2021; Wan et al., 2019). However, the use of algae in feed faces certain challenges, primarily the high costs associated with these materials and the competition with the food industry. Nevertheless, the algae production industry has been expanding in recent years, and as production processes are optimized, costs are expected to decrease (Araújo et al., 2021; Camacho et al., 2019).

This growth in algae production also presents opportunities for these companies to explore alternative applications for their products beyond traditional food uses (Araújo et al., 2021). Using whole algae, rather than extracts, has the potential to be more cost-effective for the feed sector. However, certain value-added compounds extracted from algae, like bioactive proteins originating from microalgae and macroalgal polysaccharides (e.g., fucoidan and ulvan), might also be of interest in the functional feeds market (Uma et al., 2023). To facilitate the exploration of these compounds, novel *in vitro* tools for aquaculture can enable rapid screening (Goswami et al., 2022). By leveraging these innovative tools, smaller quantities of products can be utilized for initial screening, allowing only the products with high potential to be selected for future applications. This approach promises to yield long-term benefits for the aquaculture industry, both in terms of economics and ethical considerations.

The future perspectives for finding algae-based solutions for fish health in aquaculture are full of exciting possibilities. One of the key opportunities for exploration involves the development of novel tools and technologies to assess *in vitro* functional compounds with immunomodulatory potential. This includes the development of antibodies and fish cell lines (Goswami et al., 2022), the establishment of 3D models such as organoids (Beaumont et al., 2021), and potentially, the development of organs-on-chip (Drieschner et al., 2019), complex systems that can better simulate *in vivo* processes. As researchers delve deeper into the immunomodulatory potential of algae-based compounds, the development of specific antibodies for target molecules becomes crucial. These antibodies can be used to study the interaction between algae-derived compounds and the fish immune system,

shedding light on the mechanisms through which algae influence fish health. The establishment of fish cell lines offers a valuable resource for *in vitro* experiments. Though these cell lines have certain limitations, they can be used in the first step of the screening process to select algae-derived compounds that can contribute to better functioning of fish immune cells. It opens opportunities for high-throughput screening and detailed investigations into immune responses (Goswami et al., 2022; Rocha et al., 2023).

The findings obtained from this thesis, particularly the in-depth characterization of distinct intestinal segments and the precise localization of proliferating cells, can establish a solid foundation to inform and guide future research efforts focused on the development of cutting-edge tools for European seabass studies. Additionally, the comprehension of the seabass gut microbiota's core composition and its response to dietary formulations including algae presents fundamental insights that can be integrated into novel *in vitro* tools. In this context, organ-on-chip technology represents cutting-edge *in vitro* technology where multiple compartments housing immune cells and bacteria, simulating the seabass microbiota, can be utilized to simulate the fish intestine. Such a model could function as a platform to probe the intricate interactions between immune cells and the microbiota. Furthermore, utilizing state-of-the-art methods like microfluidics, various compounds could be introduced into the system to elucidate the complex relationship between dietary components, immunity, and the microbiota (Bein et al., 2018).

By integrating these advanced tools and technologies, future research can provide a deeper understanding of the immunomodulatory potential of algae-based compounds. This knowledge will not only contribute to the development of optimized fish diets but may also lead to the creation of innovative functional feeds designed to enhance fish health and disease resistance. Moreover, fish farmers and aquaculture professionals can benefit from this research by implementing strategies and products that improve the overall health and resilience of farmed fish, ultimately contributing to more sustainable and efficient aquaculture practices. These innovative approaches have the potential to play a pivotal role in shaping the future of aquaculture and contributing to the well-being of farmed fish.

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“Humans fancy that there's something special about the way we perceive the world, and yet we live in loops, (...) seldom questioning our choices, content, for the most part, to be told what to do next” in Westworld. To break free from the repetitive cycle of life, we must strive for a future that embraces additional challenges, allows room for mistakes, and welcomes greater uncertainty.