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# Nutrition and sustainable development goal 14: Life below water

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# Nutrition and sustainable development goal 14: Life below water

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# Editorial: Nutrition and sustainable development goal 14: life below water

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**human, nutrition, health, sustainable use of resources, aquatic animals, diets, quality**

Editorial on the Research Topic
Nutrition and sustainable development goal 14: life below water

Healthy and nutritious diets are of central importance to the United Nations (UN) Sustainable Development Goal (SDG) 14 "Life under water" targets, as basis for the improved conservation and sustainable use of oceans, seas and marine resources. The availability of abundant, nutritious sources is fundamental to high quality nutrition of both aquatic animals and humans and is intricately linked to the reduction of pollution and the effects of climate change. The role of aquatic foods in sustainable healthy diets was recently revised by UN's Nutrition section (1), as a lack of sufficient strategic promotion of their dietary effects was recognized. A policy brief with recommendations was emitted by UN on basis of the 2021 discussion article (2). Aquatic foods for human nutrition have many essential properties, being some of the best sources of vitamins A, B12, D, bioactive compounds, and numerous minerals (calcium, iron, iodine, zinc, selenium, phosphorus, etc.), with high bioavailability (3, 4). Aquatic foods are also significantly different in their composition and properties from most terrestrial foods and are substantial for many coastal communities worldwide. These considerations have brought to the inclusion of the following three key messages in the published UN policy brief: Encourage diversified consumption of aquatic foods, including low-trophic aquatic foods; Ensure equitable and sustainable supply and production of aquatic foods; Democratize knowledge, data, and technologies. The following Research Topic of scientific publications contributes novel knowledge promoting Sustainable Fisheries, increasing the economic benefits from sustainable use of marine resources, and increasing research and technology for ocean health. It is organized around two main knowledge areas - Aquatic foods as marine resource for human nutrition, and Nutrition in aquaculture.

Ebrahimi et al. reviewed the methods for achieving synergistic and additive effects of natural marine bioactive compounds and extract combinations with anti-obesity, anti-inflammatory, antioxidant, and chemo preventive activities in the last two decades. While most marine bioactive combinations this far have been concentrated on potential synergies of fish oil and carotenoids, strategies to increase the number and diversity of marine bioactive combinations and develop marine-based functional foods with higher efficacy for disease prevention, are needed.

Zeng et al. examined the relationship between n-3 PUFA poor/rich seafood consumption and gout, the most prevalent inflammatory arthritis, by use of data from the National Health and Nutrition Examination Survey (NHANES) for US adults. A

dose-response analysis showed, in the female group, non-linear relationship between n-3 PUFA rich seafood intake and the risk of gout. Additionally, the study provided proof-of-concept regarding the prevention potential for n-3 PUFA rich seafood against the harmful effects of purines in gout.

The integrated approach of the Samaki Salama project intervention (Blackmore et al.) aimed to promote sustainable fishing practices and improve the nutritional status of young children <5 years of age in small-scale fishermen households in Kenya. The study provides an example of how to leverage multiple disciplines to address key challenges to human and environmental health and illustrates a pathway for scaling study innovations to other small-scale fisheries systems.

Sustainability in consumption has also been explored by Kendler et al. by studying the potential use of several underutilized marine species, namely flounder (*Platichthys flesus*), lemon sole (*Microstomus kitt*), megrim (*Lepidorhombus whiffiagonis*), plaice (*Pleuronectes platessa*), and thornback ray (*Raja clavate*), often captured as by-catch in Norway, as an opportunity to ensure future supply of high quality, sufficient fish for consumption by the ever-growing population. All five species showed remarkable nutritional quality in the distribution of digestible indispensable amino acid ratio (DIAA) and the two main n3 fatty acids - EPA and DHA.

One of the main challenges in aquaculture nutrition nowadays is the replacement of fish oil (FO) in aquafeeds, which implies identifying sustainable alternative sources of EPA and DHA, to ensure the naturally required LC-PUFA levels in marine fish.

Marques et al. evaluated different combinations of lipid sources rich in n-3 LC-PUFA, available on the market as a marine alternative to replace traditional fish oil (sardine oil), in diets for European sea bass (*Dicentrarchus labrax*). The analysis demonstrated that n-3 LC-PUFA rich sources from salmon oil, algae oil and a blend of micro and macroalgae (Algaessence Feed™) were viable solutions for the direct replacement of traditional fish oil or for preparation of combinations to fortify European sea bass.

In parallel, Mota et al. evaluated the potential of a commercial algae blend, composed of macroalgae (*Ulva* sp. and *Gracilaria gracilis*) and microalgae (*Chlorella vulgaris* and *Nannochloropsis oceanica*), in a plant-based diet (up to 6% on dry matter basis) upon the digestibility, gut integrity, nutrient utilization, growth performance, and muscle nutritional value of European seabass juveniles. The study demonstrated the beneficial effects of the commercial algae blend supplementation; however, feeding trials up to commercial-size fish are needed to fully assess its potential as feed.

The study conducted by Zhang et al. investigates by molecular approaches the effects of phenylalanine supplementation on gene regulation of growth, digestive capacity, antioxidant capability, and intestinal health of triploid rainbow trout (*Oncorhynchus mykiss*), fed with low fish meal diet.

Nguyen et al. examined the impact of different levels of dietary methionine concentrations, combined with elevated sea temperature on feed intake, brain expression of selected neuropeptides and melanocortin 4 receptor involved in appetite control in juvenile cobia (*Rachycentron canadum*). The study highlighted a significant effect of temperature and dietary methionine on appetite-related neuropeptide expression in juvenile cobia brains.

Guo et al. evaluated the potential use of Perilla, fish, or soybean oil as lipid sources in the diet of the Chinese giant salamander (*Andrias davidianus*), to fulfill the need for compound feed development. The study showed that all analyzed oils (Perilla, fish, and soybean) positively affect growth performance and specifically emphasized that Perilla oil, as fat source of *A. davidianus* compound feed, can enhance muscle quality and antioxidant capacity, boost immunity, promote lipid metabolism, and maintain liver and intestinal health.

In summary, the results from the above-mentioned studies and reviews represent a substantial knowledge contribution to: (1) establishing possibilities for increased sustainable future aquatic food consumption, e.g., through the development of novel functional foods and bioactives/ nutraceuticals with inclusion of alternative marine sources; (2), developing approaches for improved quality of cultured fish or alternative aquatic animal species, by use of novel feed sources and balanced feed diets. The integrated approach of the Samaki Salama intervention (Blackmore et al.) provides an example of how to leverage multiple disciplines to address key challenges to human and environmental health and illustrates a pathway for scaling study innovations to sustainability estimates of various small-scale fishery systems.

## Author contributions

MA: Writing – original draft, Writing – review & editing. LK: Writing – original draft, Writing – review & editing.

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The 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.

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# *Samaki Salama* – Promoting healthy child growth and sustainable fisheries in coastal Kenya: A study protocol

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**Background:** One in five young children globally suffer the consequences of stunted growth and development and millions experience deficiencies in zinc, iron, iodine, vitamins A and B12, nutrients found bioavailable in fish foods. Small-scale fisheries have the potential to generate income and augment fish consumption while being environmentally sustainable if appropriately managed. However, those engaged in small-scale fisheries are often marginalized, poor, and malnourished. The *Samaki Salama* project seeks to better understand and address these challenges through a three-arm, longitudinal matched cluster study which evaluates the impact of an integrated nutrition social marketing and modified fishing trap intervention.

**Methods:** There will be 400 small-scale fisher households enrolled from Kilifi County, Kenya and residing in communities matched on location (rural), livelihoods, and child nutritional status. The sample will include mothers and other caregivers, children 6–60 months, and fishers in the family. Applying a cluster design, the matched communities will be divided into three groups: (1) control ($n = 200$); (2) multi-component nutrition social marketing intervention to fishers, mothers, and health workers ($n = 100$); and (3) multi-component nutrition social marketing intervention plus modified fishing traps and training ($n = 100$). Primary outcomes include child growth, fish food intakes, and fisheries yield of mature fish. Secondary outcomes are diet diversity, child diarrheal morbidity, and fisheries revenue. A process evaluation will be used to monitor and ensure fidelity of intervention delivery.

**Discussion:** This study builds on a growing body of literature illustrating the effectiveness of nutrition focused social marketing campaigns to promote active engagement of participants, high compliance to the intervention, and

sustained behavior change. The second intervention element of modified fishing traps that allow immature fish to escape enables participants to act on the messaging they receive and promotes sustainable fishing through increased harvest efficiency and reduced catch of immature fish. The integrated approach of the *Samaki Salama* intervention provides an example of how to leverage multiple disciplines to address key challenges to human and environmental health and illustrates a pathway for scaling study innovations to other small-scale fisheries systems.

**Trial registration:** https://clinicaltrials.gov (NCT05254444).

KEYWORDS

**stunting, nutrition, food security, sustainability, fisheries, social marketing**

## Introduction

Human populations continue to experience nutrient deficiencies with dire health consequences associated with diminishing dietary diversity and a lack of access to certain foods. Stunted growth and development arise from these deficiencies and act synergistically with poverty to reduce the potential of societies to achieve productivity and human wellbeing. Globally, the nutrient deficiencies found widely prevalent – zinc, iron, iodine, vitamins A and B12, among others – are also those found bioavailable in fish foods (1, 2). This speaks to the need to ensure access and more equitable distribution of nutrient-dense foods such as fish across populations. Economic, social, and environmental factors act in tandem to influence access and associated nutrition security. Here we propose a study to test the effects of a multi-component nutrition-sensitive intervention to improve nutrition security along with fisheries sustainability and economic resilience in the first 1,000 days of life and beyond.

Definitions of food security commonly include access, availability, and utilization of nutritious and safe diets year-round (3). Most evidence for nutrition interventions, however, comes from trials testing nutrition-specific interventions or those with direct effects on nutrition such as micronutrient supplementation or fortified foods (4). Consequently, there is a need to build the evidence-base for nutrition-sensitive interventions that target food production and income pathways. Our project embeds nutrition objectives and activities in an intervention to increase sustainable fish production through the provision of modified fishing gear, improve household income, and increase awareness about the importance of fish for maternal and child nutrition. We specifically target small-scale fisher households who are particularly vulnerable to food insecurity and malnutrition and lack the necessary extension support and inputs to increase efficient, sustainable fish production.

Poverty and malnutrition disproportionately affect populations engaged in small-scale food production through fisheries, subsistence agriculture, or livestock production (5). Yet, small-scale production provides the majority of food in developing countries and supports the livelihoods of over 60% of the workforce in this sector (6). Of the small-scale food production sectors, fisheries are a major contributor with more than 50 million men and women employed in the sector (7). However, small-scale fisheries (SSF) are underrepresented in the dialogue around the Blue Economy and have been marginalized by large industrial fisheries, aquaculture ventures serving global markets, establishment of no-take fisheries closures to meet conservation goals, coastal development, foreign tourism, and mineral extraction (8–10). Furthermore, considerations of food and nutrition security have been largely absent from Blue Growth initiatives. Our study positions food supply and nutrition security at the forefront of promoting sustainable SSF systems in Kenya's Blue Economy initiatives through a multifaceted intervention that can be scaled to other systems, including aquaculture.

Food production often comes at the cost of the environment and biodiversity (11–13). Compared with industrial fisheries that tend to target a single species, SSF can be more sustainable and achieve greater outputs per unit effort through the utilization of more passive gear types (traps, gill nets, long lines, etc.) that minimize damage to local ecosystems (14). Small-scale fishers are also more likely to keep and utilize a wide range of the marine species that are caught (14). A more diverse catch spreads out the impact of fishing and reduces the risk of a single species collapse or extinction. However, in contexts where SSF have limited resources and gear options, which reduces the types and number of species fishers are able to catch, the level of fishing effort on local marine ecosystems can become unsustainable and stocks may collapse with concurrent harm to the environment (14–16). The potential for over-exploitation and

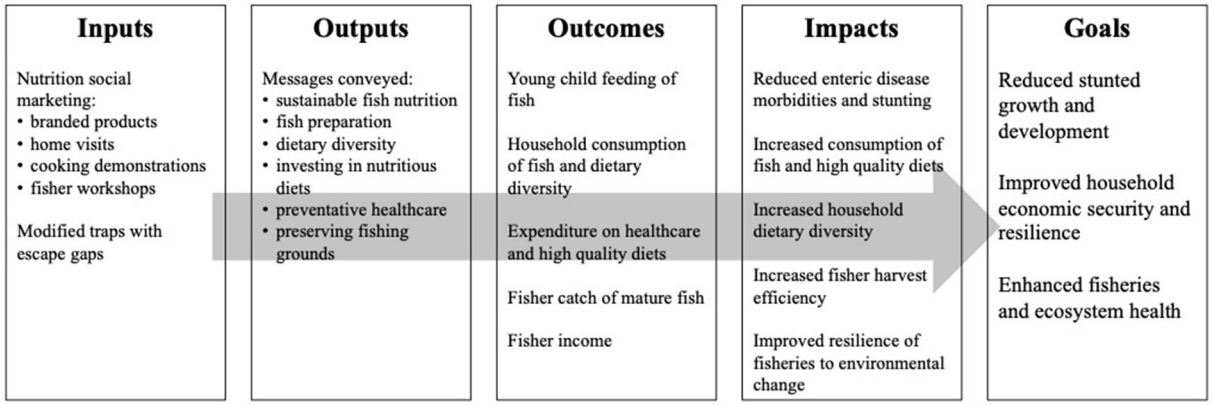


FIGURE 1
Logical framework.

biodiversity loss highlights the importance integrating marine ecosystem sustainability into interventions targeting small-scale fisher households.

Small-scale fisheries in Kenya are chronically overexploited and fish stocks are dwindling as evidenced by a four-fold decrease in marine catch since the 1980s (17). Efforts to combat declines in SSF production in Kenya have focused on improving the management of fishery resources through the establishment of Beach Management Units (BMU), gear restrictions (e.g., spear gun, beach seine) and no-take fisheries closures (18, 19). The *Samaki Salama* project continues efforts to improve fisheries production in coastal Kenya while also integrating critical dimensions of livelihood sustainability and nutrition security. *Samaki salama* means "fish security" in Kiswahili, the local language, and is used as the project name to highlight the economic, ecological, and nutritional facets of the intervention.

## Logic framework

Figure 1 illustrates the logical framework for our integrated nutrition and fisheries intervention We expect our nutrition social marketing campaign to increase consumption of fish among children under 5 and dietary diversity for households, leading to reduced enteric disease morbidities and reduced stunted growth and development. We anticipate modified fishing traps with escape gaps (to allow immature fish to leave the trap while retaining mature fish) to promote fisheries sustainability and increase fisher income, leading to greater harvest efficiency, improved resilience to environmental change, dietary diversification, and improved nutritional status through both direct consumption and indirect poverty reduction pathways.

# Methods and analysis

## Design

We aim to test the effectiveness of the multi-component *Samaki Salama* intervention as it intersects with nutrition security and fisheries sustainability in Kilifi County, Kenya. Table 1 details the specific aims and hypotheses.

The intervention will target communities matched based on location (rural), livelihoods, and child nutritional status. The matched sample will be divided into three groups: (1) control ($n = 200$); (2) multi-component nutrition social marketing intervention to fishers, mothers, and health workers ($n = 100$); (3) multi-component nutrition social marketing intervention plus modified fishing traps with escape gaps and training ($n = 100$). Although our original design was to have two comparison groups with equal numbers, formative research showed insufficient numbers of fishers currently using traps that could be recruited to use modified traps. Further, budgetary constraints precluded distributing traps to all 200 in the intervention group. Thus, we split the intervention group enabling us to test the added effects of modified traps together with social marketing. Figure 2 provides an overview of the study design and implementation process.

Both process and impact evaluations will be carried out using mixed methods. Primary and secondary nutrition outcomes include longitudinal difference-in-difference analyses of parameters – height-for-age $Z$ score, stunting prevalence, child fish food intake, child dietary diversity, and child diarrheal morbidity. Other outcomes include awareness of the social marketing campaign and knowledge transfer. Primary and secondary fisheries outcomes will be longitudinal difference-in-difference analyses in fisheries yield of mature fish and fisher income and earnings.

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TABLE 1 Study aims and hypotheses.

| Study aims | Hypotheses |
|---|---|
| 1. Determine the effects of a multi-component social marketing campaign to promote fish nutrition, dietary diversity, and food safety on child growth | **Primary**<br>Hypothesis 1: Children in combined intervention groups have increased height-for-age *Z* by 0.2 compared to children in the control<br>Hypothesis 2: Children in combined intervention groups have increased weight-for-age *Z* by 0.10 compared to children in the control<br>Hypothesis 3: Children in combined intervention groups have increased fish food intakes by 100 g compared to children in the control<br>**Secondary**<br>Hypothesis 4: Children in combined intervention groups have increased dietary diversity by 1.2 compared to children in the control<br>Hypothesis 5: Children in combined intervention groups have reduced diarrheal morbidity by 5 percentage points compared to children in the control<br>**Exploratory**<br>Hypothesis 6: Children in the social marketing + traps intervention group have increased dietary diversity and fish intake compared to children in the social marketing only group |
| 2. Measure the impact of modified fishing traps with escape gaps on catch dynamics and earnings | **Primary**<br>Hypothesis 7: Fishers in the social marketing + traps intervention group have significantly increased fisheries yields of mature fish compared to fishers in the control<br>**Secondary**<br>Hypothesis 8: Fishers in the social marketing + traps intervention group will have significantly increased earnings compared to fishers in the control |

The *Samaki Salama* intervention precludes randomization due to the high risk of spill-over effects and the limited number of trap fishers with children under the age of 5. Thus, for the impact assessment, we will conduct a matched intervention/control design to minimize selection bias and test effectiveness on nutrition and fisheries production. Steps have been taken to reduce the risk of selection bias that might arise from the matched design. A pilot study and additional formative research will be used to identify and match communities on important characteristics [socioeconomic status (SES), child nutrition, livelihoods, etc.]. This preliminary data provides additional information needed for external validity and extrapolation of findings to other small fisher households in Kenya and internationally. The longitudinal difference-in-difference design further contributes to the internal validity, accounting for residual confounding, and increases the statistical power to detect intervention effects.

## Sample size

Sample size calculations for the cluster design applied a mean −1.3 height-for-age (HAZ) for the coast region (20) and a hypothesized 0.20 effect size (21). Thus, we estimate requiring a total sample size of 400 households, which includes ∼4 clusters per arm (four villages per BMU) and 50–75 households per cluster – assuming 20% losses-to-follow-up ($\alpha = 0.05$ and 1-$\beta = 0.80$). Although this is small number of clusters, we will apply matching techniques a priori and during data analyses (e.g., propensity score matching) to better ensure internal validity.

## Setting

The study will be carried out in five distinct areas in Kilifi County, Kenya: Mayungu, Uyombo, Takaungu, Kuruwitu, and Kanamai (Figure 3). Study sites were chosen based on established relationships with the research team, receptivity to the proposed intervention, and proximity and access to marine resources. Kilifi County covers an area of 12,370 km$^2$ with a population of 1.45 million and average household size of 4.8 persons (22, 23). The population primarily relies on small-scale fishing, farming activities (raising livestock, tree-cropping, and food-crop production), tourism, and migration to urban centers for their livelihoods (22). Close to half the population lives in poverty (46.4%) and the stunting prevalence (39%) is nearly double the national average, indicating high levels of malnutrition (20, 24). Education levels are low, and the populations are marginalized with limited formal rights to the marine resources on which many of their livelihoods depend.

## Participant eligibility, recruitment, and retention

At the outset of the study, we will identify comparable communities associated with BMUs on the coast of Kilifi County using potential confounding variables: proximity to shoreline or ability to participate in the fishery; presence of no-take fisheries closures; composition of fishing gears

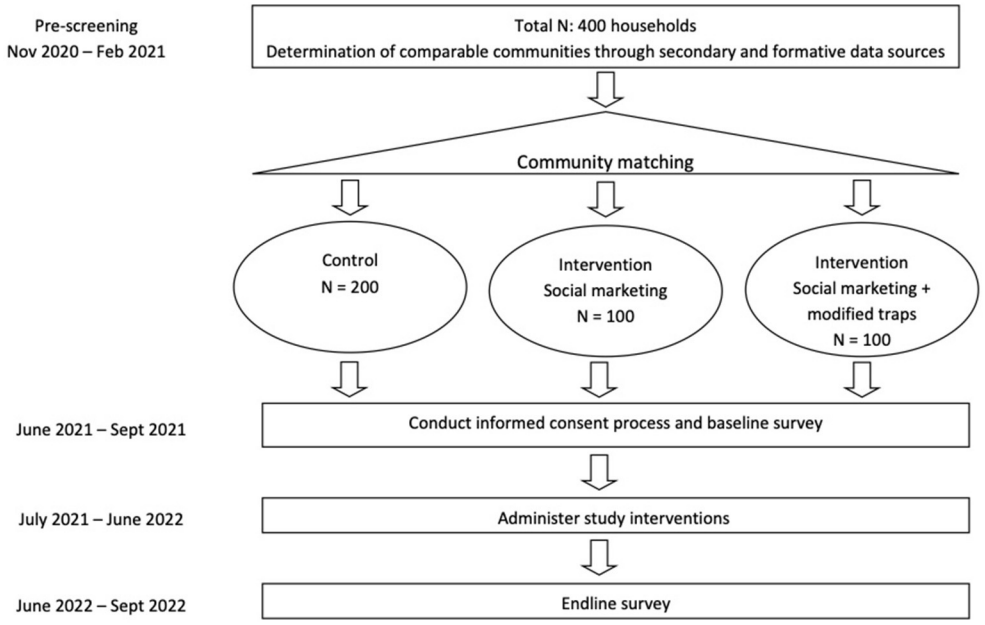


FIGURE 2
Study design.

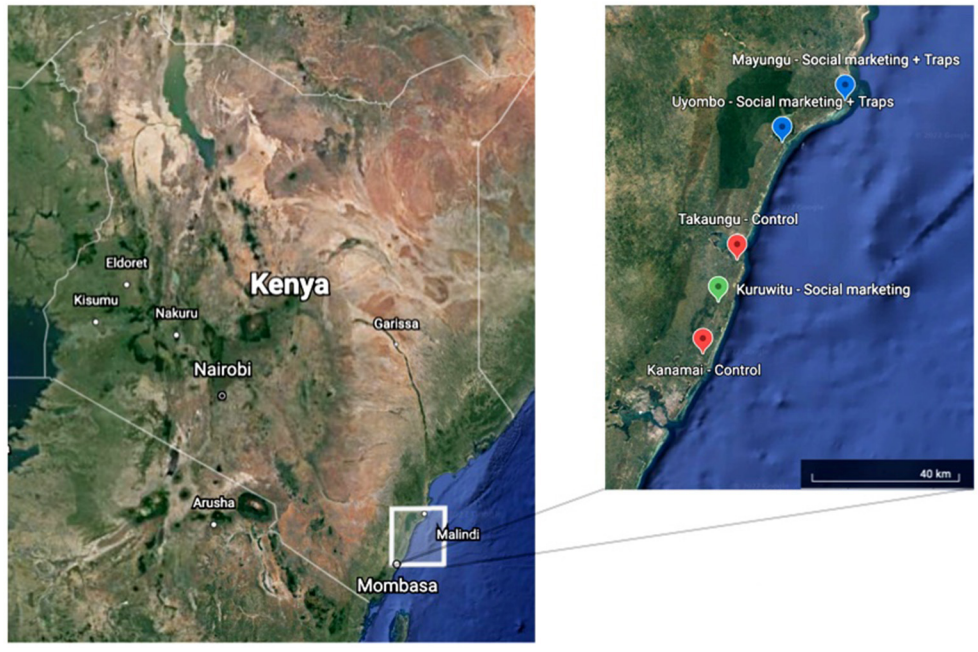


FIGURE 3
Study area (Source: Google Earth).

used; background nutritional status; usual diets; income and assets; and access to market information. We will draw on existing relationships and data from communities where formative research was previously conducted by the project team. BMU leaders and other stakeholders will be convened to first inform them of the potential project and solicit

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permission to act in partnership with BMUs to conduct the research.

To be eligible to participate in the study, an individual must meet all of the following criteria:

1. A household member works in small-scale fisheries (self-employed fishers).
2. At least one child in the household aged 6–60 months.
3. Provision of signed and dated informed consent form.
4. For children, informed assent and parental informed consent to participate in the study.
5. Stated willingness to comply with all study procedures and availability for the duration of the study.

An individual who meets any of the following criteria will be excluded from participation in the study:

1. No household member works in small-scale fisheries (self-employed fishers).
2. No child in the household aged 6–60 months.
3. Declines to sign the informed consent.
4. Index child is severely malnourished.
5. Lives outside the study area.
6. Participation in another nutrition or fisheries intervention study within April 2021 - June 2022.

We anticipate a total sample size of 400 small-scale fisher household units (mother, father, and child 6–60 months). There will be 100 in each intervention group (social marketing and social marketing plus modified traps) and 200 in the control group. Table 2 provides a summary of the total number of individuals and anticipated demographics.

Participant recruitment and enrollment will be conducted by the two Kenyan partner universities (Egerton University and Pwani University). Pwani University, located in Kilifi County and on the coast, is well-positioned to be in continuous interaction with community stakeholders. The Kenya P.I. from Pwani has established relationships in Kilifi over many years of working there and teaches many university students from the neighboring fishing villages. Additionally, the US Co-P.I. from the University of Rhode Island (URI) has worked in this area for over 10 years conducting research on coral reef fisheries management and has long-term working relationships with fishers and BMU leaders. The Kenya Co-P.I. from Egerton University was involved in formative research along the coast and has made contacts with local health care workers and clinics for collecting data and measuring nutrition outcomes.

Recruitment efforts will begin with consultations with key community stakeholders including BMU leaders and board representatives; health care workers in local clinics; community health workers (CHVs); religious leaders; municipal administrators. With their agreement, communities will be matched based on the closest set of characteristics and assigned to control and intervention groups.

TABLE 2 Sample size and demographics.

| | Group 1: Control (n = 200) | | Group 2: Social marketing (n = 100) | | | Group 3: Social marketing + modified traps (n = 100) | | |
|---|---|---|---|---|---|---|---|---|
| **Group** | **# of individuals** | **Anticipated sex** | **Group** | **# of individuals** | **Anticipated sex** | **Group** | **# of individuals** | **Anticipated sex** |
| **Total sample size: 400 total households** | | | | | | | | |
| Mother/caregiver | 200 | Female | Mother/caregiver | 100 | Female | Mother/caregiver | 100 | Female |
| Fisher | 200 | male | Fisher | 100 | Male | Fisher | 100 | Male |
| Children 6–60 months | Minimum of 200 | Mixed | Children 6–60 months | Minimum of 100 | Mixed | Children 6–60 months | Minimum of 100 | Mixed |

Retention efforts will be primarily carried out in tandem with the social marketing campaign. Activities to increase participant engagement – both in control and intervention groups – will include meetings, social gatherings, and project materials (e.g. T-shirts, flyers, lifejackets). These efforts are modeled from the Lulun Project in Ecuador (25) and adapted to the Kenyan context should help retain participants.

## Interventions

The 12-month *Samaki Salama* intervention introduces practices and technologies that build on existing community assets and expressed needs and preferences of small-scale fishers and their households. The first component of the intervention package, nutrition social marketing, is a novel approach to increase nutrition awareness across multiple stakeholders that draws on psychology, marketing, and communications disciplines (25). Distinct from conventional nutrition education interventions that tend to use more didactic approaches and standardized materials, this strategy draws heavily on contextual factors to identify key messages and delivery platforms. Nutrition-focused social marketing campaigns have been shown to promote active engagement of participants throughout the trial, high compliance to the intervention, sustained behavior change, and low losses to follow-up (26). The effectiveness of social marketing increases if targeted messages are repeated and delivered across different platforms (27) and previous studies indicate that 6 months of animal source food nutrition messaging may not be enough to sustain impact (28). Therefore, we propose a 12-month intervention period.

The nutrition social marketing approach will be developed in close collaboration with the social and behavioral change group at USAID Advancing Nutrition. The focus is on promoting four priority behaviors among infants, young children and women of reproductive age in SSF households: (1) caregivers feed fish to young children 6 months to 5 years daily; (2) caregivers feed an age-appropriate diverse diet, including fish, to children 6 months – 5 years daily; (3) caregivers wash hands and the child's hands with soap or ash before feeding; (4) fathers reserve and take home a small portion of fish for child each day. The social marketing plan and audience analysis, including all materials, messaging, and delivery platform relies on formative research conducted in the study area and piloting of materials prior to implementation. As shown in Table 3, messaging centered on the four priority behaviors will be delivered through diverse channels to mothers/caregivers, fathers, and leaders/members of local institutions. The plans to engage these participants groups reflects a multi-level approach to support improved feeding and care behaviors through individual, family, and community level change.

*Home visits* are a key component of the social marketing campaign and will occur at a three time points (3, 6, and 9 mo.) in both intervention groups. All homes in the intervention groups ($n = 200$) will be visited at each time point. Visits every 3 months will allow the field team enough time to complete one round of visits before starting the next round. Three visits per household is the maximum the field team anticipates being able to complete within the 12-month intervention. During visits, the team's nutrition education specialists will actively engage with the caregiver and child/children to build a positive relationship, understand their individual needs, and foster change. The suite of social marketing materials available to the field team will be the same for all intervention households but we anticipate that the exact information and materials provided will be tailored to meet the needs of the individual household and child/caregiver dyad. The nutrition team will discuss and identify illnesses before introducing age and stage-specific child feeding and hygiene habits (or recommendations) and help caregivers identify next steps and agree on actions geared toward improving feeding and hygiene practices and better health care. The overarching message caregivers will receive is that fish can be a critical source of nutrients for their child and paying special attention to their child's growth and development now has life-long benefits. Discussions, agreements, and actions taken will be

TABLE 3 Social marketing campaign activities and materials.

| Activity | Delivered by | Branded products |
|---|---|---|
| **Mothers/caregivers** | | |
| Personalized home visits | Staff (Home visitors); CHV | Reminder poster on home actions<br>Letter to fathers about home action<br>Calendar with child growth visual<br>T-shirts<br>Khanga cloth<br>Story book |
| Cooking demonstrations | Staff; CHVs | Menu game about nutrition foods for children |
| **Fathers/fishers** | | |
| Workshop series at fish landing sites | Staff | Commitment letter for fishing and home actions<br>T-shirts<br>Stickers<br>Life jackets |
| **Institutions** | | |
| BMUs orientations | Staff | Banners<br>Posters<br>Stickers |
| Health worker orientations | Staff, CHVs | Posters<br>Stickers<br>Bags |

documented and tracked by the nutrition team. Local CHVs in both intervention and control communities will be trained to conduct the home visits so caregivers will have a reliable source of information and support even after the project is completed.

Fifteen *cooking demonstrations* will be conducted in intervention communities with an anticipated 10–15 caregiver participants and their children per demonstration. The demonstrations will provide an overview of key nutrition concepts, important nutrients found in fish, and different approaches for fish preparation. Participants will be asked to plan a meal and snacks for their children using a "star foods" menu game and work together to prepare a fish centered meal. Questions will be asked at the beginning and end of the demonstration to better understand participants' nutrition knowledge, what they gained from the demonstration, and feedback for improving future demonstrations. Demonstrations will be conducted in local community spaces identified by the field team.

The project nutrition team will work together with the fisheries team to conduct a series of 10 *fisher workshops* at local BMU offices with fishers in both intervention groups. Table 4 provides a summary of the themes and topics that will be covered at each workshop.

Fisher training, gear modification, and fishing trap distribution constitute the second piece of the intervention. The use of fishing gears modified to decrease juvenile catch has been shown to lead to greater catch diversity and improve the economic value of fishes (16). Second, fishers can gain a competitive advantage when using new gears by fishing new habitats to catch previously targeted species in novel ways (15). This can reduce the impact of fishing on the environment, and in the case of coastal Kenya, coral reefs. Last, gear modifications have been shown to improve harvest efficiency and promote sustainable fish populations by selecting for mature individuals while at the same time improving fisher revenue (29). Using these rationales, our intervention targets fishers using basket traps and provides them with traps modified with escape gaps so immature fish can escape. Trap distribution will occur at landing sites utilized by participating fishers and at local BMU offices. The fishers will also receive training on how to properly maintain the traps they receive. We hypothesize this type of intervention will reduce fishers' dependence on immature fishes, as well as buffer them from potential market variability (30) and enable them to be more resilient to environmental change (31).

## Data collection and management

Data collection will be conducted by the research staff at the study site under the supervision of the Kenyan investigators. A household and fisher survey will be used to gather data on demographics, socioeconomics, hygiene, sanitation, mother and child health, diet, anthropometry, COVID livelihood impacts, household decision making, awareness/knowledge of fish consumption, and fishing characteristics at baseline and endline. Dietary intakes will be measured using a Kenya-specific semi-quantitative food frequency questionnaire (FFQ) (32). The survey will be developed using the Research Electronic Data Capture (REDCap™) platform and collected electronically using password protected tablets. To assure the quality of data entry the survey will utilize REDCap™'s built-in data validation. The team will also be able to track access of data, instruments, and reports through an electronic audit trail. To minimize missing data the field team coordinators will review all REDCap™ records for completeness prior to uploading the data to the secure REDCap™ server hosted by Washington University in St Louis. If any issues are found the coordinators will follow up with the enumerator responsible for the data entry. Fisheries catch, take home amount, and income data will be collected at least four times monthly at landing sites using paper forms that are then entered into Microsoft Excel by the fisheries field team and stored on password protected computers. Excel data entry is mediated with built-in validation to a given list of marine fish species found locally. All paper forms will be stored in a locked filing cabinet when not being processed. Data will be screened for completeness and consistency on a bi-weekly basis, with archival data stored in the password-protected Box cloud storage platform, and if any issues arise, the research team will follow up with the data collectors.

A process evaluation will be used to monitor and ensure consistent administration of the intervention (fidelity of delivery), adoption, and sustainability, three key implementation outcomes (33). The primary focus is on documenting the transition from inputs [nutrition social marketing and fisher support (modified traps)] to the anticipated outputs, outcomes, and impact (Figure 1). Systematically tracking, documenting, and assessing this part of the impact pathway will allow for a more nuanced understanding of the implementation process, how and why the intervention does/does not have the anticipated impact, and facilitate future replication. Mixed methods will be used to collect information along the impact pathways. Table 6 provides an overview of the implementation process outcomes, methods, and data types.

All data collected for this study will be stored on the REDCap™ platform and on Box, a secure, Health Insurance Portability and Accountability Act (HIPAA) and Family Educational Rights and Privacy Act (FERPA) compliant data storage and sharing online platform.

## Study measures

To evaluate intervention impacts, we will assess primary outcomes of child growth along with fish food intakes, and fisheries yield of mature fish. Secondary outcomes of interest, which are also hypothesized to serve as mediating factors

TABLE 4 Themes and topics of fisher workshops.

| Workshop theme | Intervention group | Discussion topics |
|---|---|---|
| "We provide for our families" | Social marketing + modified traps | What it means to fishers to provide for their families |
| | | Challenges they face providing for their families |
| | | Benefits of using modified traps for income, their families nutrition, and fishery sustainability |
| | | Commitment to using modified traps |
| | Social marketing | What it means to provide for their families |
| | | Challenges they face providing for their families |
| | | Stages of fish growth |
| | | Activity to estimate mature size of common fish |
| | | Reasons to focus on catching more mature fish |
| | | Commitment to catch more mature fish |
| "We protect our families and communities" | Social marketing + modified traps | Check in on use of modified traps |
| | | Hopes they have for their children |
| | | Actions they can take to protect their children's health |
| | | Encouraging each other to protect their children's nutrition |
| | | Commitment to bring home some of their catch for their children |
| "We bring our BMU community" | Social marketing + modified traps | Check in on use of modified traps |
| | | Refresh knowledge of child nutrition |
| | | Discuss changes they have noticed in the areas where they fish (environment, fish quantity, fish types etc.) |
| | | Importance of sharing the information they have received with other fishers |
| | | Activity to develop a persuasive argument to protect the places where they fish and their children's nutrition |
| | | Commitment to share what they have learned with new fishers and friends |

between the intervention and primary outcomes, include indicators for child health and diet (dietary diversity, prevalence of diarrhea) and fisheries earnings. Measurement of the targeted nutrition endpoints will occur at two timepoints as part of a household level survey. The household surveys will also collect information on other relevant measures and potentially confounding factors including household expenditures, and household decision-making. Measurement of fisheries focused endpoints will occur at regular intervals over the course of the 12-month intervention at commonly utilized fish landing sites within the study area. Table 5 summarizes measures that correspond with outcomes of interest.

## Primary outcomes

### Child growth

Anthropometric measures (length/height, weight) will be collected from children and mothers/caregivers at baseline and endline. The Seca Model 874 (Digital) 440 lbs. × 0.1-lb. resolution and the ShorrBoard® stadiometer will be used to collect weight and length measures, respectively. Measures will be converted to weight-for-age *Z* (WAZ), length-for-age *Z* (LAZ)/HAZ, weight. HAZ and WAZ will be generated using World Health Organization (WHO) (34). The Stata Macro available from WHO will be run to derive the indicators using data on child age in months, sex of the child, and child height/weight. Outliers above HAZ/WAZ $> 6$ or HAZ/WAZ $< -6$ will be removed.

### Dietary assessment/fish food intakes

Dietary intakes will be measured using a Kenya-specific semi-quantitative food frequency questionnaire (FFQ) (32). A comprehensive list of foods consumed in Kenya, and specifically along the coast, will be compiled along with ingredients in common dishes. This will be integrated into the survey as an FFQ for 24-h intakes of women of reproductive age, youth, and children ages 6–60 months. Particular attention will be given to fish foods and other animal source foods which will be asked as 24 h and 7-day recalls. Findings from the FFQ will later be converted to the Feed the Future (FTF) indicators of minimum dietary diversity for women and young children. Finally, infant and young child feeding practices (IYCF) practices will be assessed in accordance with the FTF minimum acceptable diet indicator.

### Fisheries yield of mature fish

Trained field enumerators will record fish catches at landing sites (fishery-dependent data). The field team will ask permission to count and weigh a fisher's catch when they return from fishing.

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TABLE 5 Study outcomes.

| Construct | Methods | Indicator | Timepoints |
|---|---|---|---|
| **Primary outcomes** | | | |
| Child growth | Anthropometric measures of height and weight | Length-for-age *Z* score (LAZ)<br>Weight-for-age *Z* score (WAZ)<br>Weight-for-length *Z* score (WLZ) | Baseline, 12 mo. |
| Fish food intakes | 24-h intake survey | Fish food intakes in g | Baseline, 12 mo. |
| Fisheries yield of mature fish | Fish catch survey at landing sites | Monthly catch per unit effort and fish size distribution | 5–10 times per month at randomly stratified days over the course of 12 months |
| **Secondary outcomes** | | | |
| Child diet diversity | 24-h intake survey | Diet diversity score | Baseline, 12 mo. |
| Diarrheal morbidity | HH survey, recall by mother during home visits | Reported acute diarrhea in the last 2 weeks | Baseline, 3, 6, 9, and 12 mo. |
| Fisheries earnings | Fish catch survey at landing sites | Earnings [in Kenyan shillings (Ksh)] per fishing trip | 5–10 times per month at randomly stratified days over the course of 12 mo. |

TABLE 6 Process evaluation outcomes.

| Outcome | Methods | Data collected | Timepoints |
|---|---|---|---|
| Fidelity | Counts of activities/events<br>Activity/event sign-in sheets<br>15–20 semi-structured interviews with caregivers and fishers | Age of the participant/s, sex (M/F), date, material that was distributed<br>Project materials viewed<br>Messages that they are aware of<br>Perceptions of the materials and messages<br>Challenges understanding the messages<br>What worked well, what could be improved | Rolling basis as activities/events are conducted<br>6–9 mo. |
| | Semi-structured discussions pre/post activities | What was learned<br>What worked well, what could be improved | 6–9 mo. |
| | Field team reports | Reports detailing implementation of activities | 6 mo., 12 mo. |
| | Household/fisher survey | Awareness of nutrition messaging | Baseline, 12 mo. |
| Adoption | Caregiver home visits | Intentions to try, actions since last visit, observations | 3, 6, and 9 mo. |
| | Household/fisher survey | Knowledge transfer linked to the social marketing campaign | Baseline, 12 mo. |
| | Meetings with fishers | Self-reported use of traps, intentions to try | 6–12 mo. |
| Sustainability | 15–20 semi-structured interviews with key informants | What they know about the intervention<br>Perceptions, feasibility for maintaining it once the intervention ends | 12 mo. |

Upon consent, the enumerators will identify the fish species or genus level and measure the lengths of a sub-sample of the individuals ($n = 20$). Sampling will occur at least four times per month at randomly stratified days, as determined by the moon phase and considering dominant gear types. Monthly catch per unit effort (CPUE) will be determined as the mean daily catch multiplied by the fishing days per month. We will also evaluate species-specific length-frequency distributions to determine the yield of mature individuals. Sustainable yields will be determined by comparing the initial yields vs. the rate of change of yields for each landing site or BMU, based on average length of catch for given species.

## Secondary outcomes

### Child diet diversity

The Child Dietary Diversity Score (CDDS) will be calculated using the total number of food groups reported in the food

frequency intake portion of the survey. We will use WHO defined food groups: (1) grains, roots, and tubers; (2) legumes and nuts; (3) dairy products (milk, yogurt, cheese); (4) flesh foods (meat, fish, poultry, and liver/organ meats); (5) eggs; (6) vitamin A rich fruits and vegetables; and (7) other fruits and vegetables. If new indicator guidelines are released before data analysis activities of this project are undertaken, we will apply the new definition.

*Diarrheal morbidity* will be calculated using a standard 2-week recall conducted during the household survey and home visits. Questions will assess diarrheal severity including the frequency of diarrhea in the children, presence of blood or fever, use of antimicrobials, and requirement for additional medical care at a clinic or local provider. This data will be used to estimate indicators for acute diarrhea (3 or more liquid or semi-liquid stools in a 24-h period over the last 2 weeks) and persistent diarrhea (lasts 14 days or longer).

### Fisher revenue

During fisheries yield data collection, enumerators will also ask fishers about their operational costs and the revenue generated from selling the fish. Fisher revenue will be represented as Kenyan shillings (Ksh) per fishing trip. These questions will be informed by cultural norms and objects such as food and equipment used as currency when Kenyan shillings cannot be estimated (e.g., bags of rice). Comparisons will be made at the landing site or BMU level to measure the change in earnings pre- and post-intervention as described by Wamukota et al. (35).

A range of other variables will be assessed to control for cofounding factors associated with the cluster design. These variables include but are not limited to child illness (a standard 2-week recall on infectious illnesses including cough, rhinorrhea, fever, and rash), household consumption and assets, and household decision making [Likert scale instrument that captures common domains of decision-making including purchasing decisions; decisions regarding service use (health, education); decisions regarding children's diet, health and education].

## Process evaluation

As shown in Table 6, the process evaluation will examine three key implementation outcomes: fidelity, adoption, and sustainability. The *fidelity* outcome will capture the degree to which the intervention was implemented as described in the study protocol, adherence over the course of the intervention, and the quality of program delivery (33). Methods for collecting and documenting implementation fidelity include activity/event counts, semi-structured interviews with intervention participants, reports from the field team, and a baseline/endline survey of caregivers and fishers that assess awareness and knowledge transfer associated with the social marketing campaign.

*Adoption* will focus on better understanding participants intention to try to actualize the information they receive (33). During home visits with caregivers the research team will observe what changes the mother/caregiver has made and their intention to try to act on the messaging in the future. Meetings with fishers will gather similar information as well as asking fishers to report on their use of the modified traps.

The *sustainability* outcome is intended as an initial assessment of local institutions interest and ability to maintain the intervention once it has been completed. Semi-structured interviews with BMU officials, heads of local health clinics, CHVs, and other relevant local government representatives will be used to assess what they know about the intervention, their perceptions of it, and institutions interest and potential for maintaining.

# Analysis

## Primary and secondary outcomes

Generalized linear regression modeling (GLM), allowing for non-normal distributions, will test the continuous outcomes of HAZ, WAZ, child dietary fish intake, child dietary diversity score, fisheries yield, and fish earnings. As a difference-in-difference analyses, change variables for each outcome will be examined (difference between baseline and endline). For the binomial outcomes of diarrhea morbidity (and other outcomes of stunting and underweight), we will estimate prevalence ratios by the GLM modeling with robust Poisson. If stunting prevalence in this population exceeds the acceptable threshold for use of odds ratios (0.2105), prevalence ratios (PRs) will be used to examine the intervention effect and were considered analogous to relative risk in this longitudinal study. The robust Poisson, with a classic sandwich estimator to correct the inflated variance of standard Poisson, is less affected by outliers.

To test for intervention effectiveness, the two intervention groups will be combined for all hypotheses except the secondary outcome of increased fisher earnings and exploratory hypothesis for differences between social marketing and social marketing + traps intervention groups. Regression models will be adjusted for potential confounding factors including age, sex of the child, corresponding baseline measures, and others found to differ significantly between the trial groups (e.g., maternal education). For fisheries yield, confounding factors will be used to adjust regression models, such as: water temperature, fishing ground area, coral cover, and seasonality. The $P$ significance value for Type I error (and one-tailed test) will be $P < 0.05$ and corresponding 95% confidence interval. Diagnostics for regression model assumptions, structure and observations will be applied, and corrective procedures applied as necessary. If selection bias is widely detected with important differences

across intervention and control communities, we will apply propensity score analyses (36). Data analyses will be performed with Stata software (version 16.0; StataCorp, College Station, TX) and R (4.1.2).

### Sub-group analysis

We plan to conduct sub-group analyses for both the primary and secondary endpoints based on child age (6–24, 25–48, and 49–60 mo.) and baseline anthropometry (HAZ/WAZ $< -2$ and HAZ/WAZ $> -2$). The justification for this is based on the evidence showing that these characteristics may influence the response effect. Younger children growing more rapidly may show greater response in HAZ. As well, children stunted at baseline may also show a greater response to the intervention.

### Process evaluation

A range of approaches will be used to analyze the data collected as part of the process evaluation. NVivo software will be used to code and analyze the qualitative data from the semi-structured interviews, discussions, home visit notes, and observations. A directed content analysis approach will be used with two rounds of coding (37). Phase one of the process will be closed coding using a codebook developed from the interview guides. A second round of open coding will be used to clarify any of the new ideas that were identified in phase one. Once open coding has been completed, code mapping will be conducted, and codes will be grouped into hierarchies to organize evidence as themes emerge. Throughout this process, the research team will document reflections on the content of the interviews. This documentation along with the notes of the field research coordinator will be analyzed to capture insights and possible lines of additional inquiry. Counts of actual activities and events will be compared with the project workplan to assess implementation fidelity and coverage. Differences between baseline and endline awareness and knowledge transfer captured in the household/fisher survey will be analyzed using R (4.1.2). An anticipated output of the analysis is a paper that details the implementation process, challenges that were faced, successes, and lessons learned for future replication.

## Discussion

This paper describes the study protocol for *Samaki Salama*, a three-arm longitudinal matched cluster study which tests the effectiveness of an integrated intervention to address malnutrition and its intersections with nutrition security and fisheries sustainability in Kilifi County, Kenya.

Our study offers an exciting opportunity to contribute to the nutrition-sensitive intervention evidence base. This "research for development" project can serve as a model for other programs in Kenya and globally for support of sustainable small-scale fisheries production and food security. Strategically positioned in Kenya, it offers new prototypes of support to entire small fisher households, marine biodiversity-nutrition linkages, and the application of scalable technologies. The escalating negative impact of climate change on the health and livelihoods of small-scale producers amplifies the need for this type of integrated approach and programming.

There is a growing body of literature illustrating the effectiveness of nutrition-focused social marketing campaigns to promote active engagement of participants, high compliance to the intervention, sustained behavior change, and low losses to follow-up. This study is designed to build on this literature and expand it to social-ecological contexts where there has been little to no application.

Developing and implementing a study during the COVID-19 pandemic adds an additional layer of challenges to any type of applied research. As part of the process evaluation the research team will methodically document the implementation process to facilitate replication and translation to similar SSF systems and clearly convey study adaptations necessitated by the pandemic.

Many well-intentioned interventions fall short on delivering their intended impact. Due to a lack of transparent systematic mixed methods evaluations the reason/s for limited success are often unclear and mistakes are repeated. This study can serve as an example of design, implementation, and evaluation approaches that offer clear lessons learned for future interventions and facilitate sustained change.

## Ethics statement

The studies involving human participants were reviewed and approved by the Human Resource Protection Office of Washington University in St. Louis (# 202101019) and the Pwani University Ethics Review Committee (#ERC/EXT/003/2020R). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

## Author contributions

LI, AW, EK-M, MC, and AH conceptualized the study. IB and LI wrote the manuscript with support from AW, EK-M, AH, CL, RC, MKC, and LS. IB and CL designed the process evaluation. LI and EK-M developed the nutrition methods and statistical analysis plan. AW and AH developed the fisheries methods. IB, RC, CS, FM, CC, and JC developed the data collection and management systems for the study. LS developed the materials and plan for the social marketing campaign. AW, EK-M, CS, FM, CO, CC, and JC provided technical guidance

for implementing the study in Kenya. All authors participated in the drafting of this manuscript and have read and approved the final manuscript.

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## Conflict of interest

Author LS was employed by JSI Research & Training Institute, Inc.

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.

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# Combination of marine bioactive compounds and extracts for the prevention and treatment of chronic diseases

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**Background:** In recent years, marine-based functional foods and combination therapy are receiving greater recognition for their roles in healthy lifestyle applications and are being investigated as viable and effective strategies for disease treatment or prevention.

**Aim of the review:** This review article presents and discusses the relevant scientific publications that have studied the synergistic and additive effects of natural marine bioactive compounds and extract combinations with anti-obesity, anti-inflammatory, antioxidant, and chemopreventive activities in the last two decades. The paper presents the mechanism of action and health benefits of developed combinations and discusses the limitation of the studies. Furthermore, it recommends alternatives and directions for future studies. Finally, it highlights the factors for developing novel combinations of marine bioactive compounds.

**Key scientific concepts of review:** Combination of marine bioactive compounds or extracts affords synergistic or additive effects by multiple means, such as multi-target effects, enhancing the bioavailability, boosting the bioactivity, and neutralizing adverse effects of compounds in the mixture. For the development of marine-based combinations, there are key points for consideration and issues to address: knowledge of the mechanism of action of individual compounds and their combinations, optimum ratio and dosing of compounds, and experimental models must all be taken into account. Strategies to increase the number and diversity of marine combinations, and further development of marine-based functional foods, are available. However, only a small number of natural marine bioactive combinations have been assessed, and most research has been focused on fish oil and carotenoid synergy. Therefore, more research and resources should be spent on developing novel marine bioactive combinations as functional foods and nutraceuticals.

KEYWORDS

**marine bioactives, marine nutraceutical combination, antioxidant, anti-inflammatory, anticarcinogenic, anti-obesity**

## Introduction

Chronic non-communicable diseases, such as obesity, diabetes, arthritis, cancer, and cardiovascular diseases, are increasing worldwide. The increase is mainly linked to changes in dietary habits. Increased awareness of the link between food intake habits and health has led many people to seek functional food options in their meal plans. Functional foods are defined as foods with additional or improved benefits beyond the normal nutritional value. This additional value could reduce the risk of chronic diseases. Nutraceuticals may also be extracted from foods to develop capsules or tablets as accessible supplements to dietary intake. The food ingredient market is rapidly growing, and a new source of bioactive substances is needed for functional food industries (1, 2).

Marine microbiota and fauna are valuable sources of bioactive compounds for the food and pharmaceutical industries. Bioactive compounds can be isolated from macroalgae (seaweeds), microalgae, echinoderms, crustaceans (for example, crayfish, crab, shrimp, and lobster), cephalopods (such as squid, cuttlefish, and octopus), mollusks (including mussel, clam, oyster, scallop, abalone, snail, and conch) and fish (3). So far, more than 36,000 compounds have been isolated from marine micro-and macro-organisms. The most widely isolated and researched marine bioactive compounds include carbohydrates, pigments, polyphenols, peptides, proteins, essential fatty acids, vitamins, and minerals. Marine compounds have antioxidant, anti-thrombotic, anti-coagulant, anti-inflammatory, anti-proliferative, anti-hypertensive, anti-diabetic, and cardio-protective activity; therefore, they can be used as active ingredients in functional food, nutraceuticals, dietary supplements, prebiotics, and pharmaceuticals (3, 4). Significantly, such bioactive compounds can minimize chronic non-communicable disease risk by reducing the onset of inflammation and oxidation (5).

Natural foods typically have low concentrations of nutraceuticals. As a result, large quantities of food need to be ingested to achieve the desired results in the human body (6). However, excessive consumption of certain bioactive compounds might have toxic effects or interact with medications (7). Additionally, when functional food is taken orally, most bioactive compounds are affected by the conditions of the gastrointestinal system, such as pH, metabolic enzymes, or normal microbiota of the gastrointestinal tract, which can reduce or eliminate their effectiveness or bioavailability (8). These issues can be solved by the concept of combinations which forwards the premise that disease prevention and/or treatment activities resulting from the combined impact of various compounds (influencing multiple mechanisms of action) are more successful than those caused by an individual compound that participates in only a single molecular mechanism. In addition, combinations of bioactive compounds can reduce the toxicity and side effects of the individual compounds (6).

The number of publications on the combination of marine bioactive compounds has increased in the last decade. These combinations include two main categories: "bioactive compound-drug combinations" and "natural bioactive compound combinations." Overall, 26 keyword combinations were used (a total of 4,384 articles were screened), and out of 4,384 articles, 29 articles were included in this manuscript. The rest of the articles were found by citations or by reading the suggested related articles. In the first category, marine bioactive compounds are combined with pharmaceuticals to support drug therapy (9). In contrast, in the second category, the combinations are composed of only naturally occurring bioactive compounds. Most studies focus on the first category. Although marine bioactive compounds have received high interest due to their therapeutic capability, studies on the second category remain sparse (Figure 1).

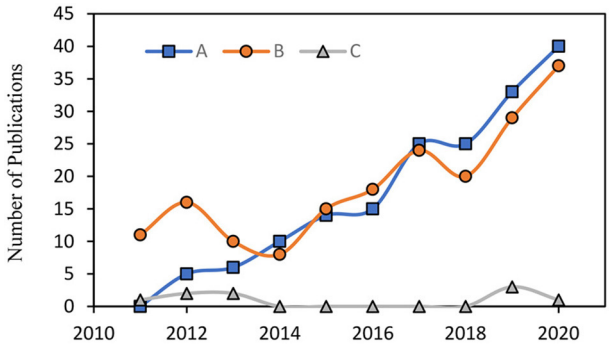


FIGURE 1
The number of publication featuring (A) "marine bioactive compounds" + "combination" in Scopus. (B) "marine bioactive compounds" + "combination" in PubMed. (C) "natural marine bioactive compounds" + "combination" in Scopus & PubMed & Google Scholar.

Over the last two decades, several review articles have investigated different combinations of phytochemicals and plant extracts on outcomes of disease prevention or treatment (10–17). To our knowledge, no review article has discussed and summarized the natural bioactive compound combinations. The main objective of this review article is to provide an overview of the scientific evidence published in the last two decades on the interaction effects of combinations of marine bioactive compounds and extracts on the treatment and prevention of various chronic non-communicable diseases. The studies related to bioactive compound-drug combinations and drug therapy are not included in this paper. The first part of this review canvasses the general mechanisms of interaction and the advantages of the combination strategy. The second part of the review discusses the health benefit of the combination of marine bioactive compounds and extracts for preventing or treating chronic diseases and elaborates on types and mechanisms of interaction. The final part of the review examines the factors for developing new combinations of marine bioactive compounds and the future research direction in the specialty.

## Underlying mechanisms of interaction and assessing combination effects

The biological effects of bioactive compound mixtures can be higher or lower than the summative effects of each component. The interactions between multiple agents are classified as potentiation, addition, synergy, or antagonism. In potentiation interaction, the combination of the two active and non-active compounds has a more significant effect than that of the single active ingredient, where the involvement of the inactive compound improves the efficacy of the active compound (18). Some natural compounds may not possess specific effects themselves but increase the solubility, absorption, distribution, or metabolism *inappropriate*

*concentration of bioactive compounds in*. Therefore, the combinations have beneficial effects by increasing the bioavailability of active compounds (16). If each component of the combination is active, it can produce an additive, synergistic or antagonistic effect. In additive interactions, the overall effect is equal to the sum efficacy of single components of the combination. If the combined effect is greater than adding individual compounds, the interaction is synergistic. However, if the combined effect is less than the addition of individual compounds, the interaction is considered antagonistic (18). A multitude of mechanisms can result in synergies, such as multi-target effects (pharmacodynamic synergism), modulation of drug transport, permeation, and bioavailability (pharmacokinetic synergism), and elimination of adverse effects of bioactive compounds (16).

The combination response observed in studies must be compared to an accepted reference model to determine the type of interaction. The most popular model is the combination index (CI) which is commonly used in both chemical- and cell-based studies. It was first established to evaluate the interactions between different drug combinations. Lately, CI has been used in food extracts and bioactive compound evaluation (17). The CI quantitatively calculates the combinatorial effect, and there is no limitation for the number of individual compounds in the combination. It is defined in the Equation as follows:

$$CI = \frac{d1}{Dx1} + \frac{d2}{Dx2}$$

d1 and d2 are the respective combination doses of drug one and drug two that produce an effect x, and Dx1 and Dx2 are the corresponding single doses for drug one and drug two that result in the same effect x. If the CI is $<1$, the interaction is a synergistic effect, while CI values higher than 1 indicate antagonism. A CI value equal to 1 is an additive effect. Another reference model is isobologram or Isobole method. It is a graphical procedure that assists in representing the trends of combination responses. The type of interaction depends on the position of the dose of combination on the "iso-effect" linear line (Figure 2) (19). However, this method is rarely used to evaluate bioactive compound interactions (17). Besides the CI and Isobole, other methods can be used to evaluate the combined effects, such as curve-shift analysis and universal surface response analysis. These two methods yield a statistical estimate of differentiation between synergy, additivity, and antagonism. In general, all four mentioned methods provide complementary information (20). In some studies, the result of the interaction is determined as synergistic or additive, but no method has been used to analyze the interaction mode. In this review, these outcomes are referred to as potential synergy or additive.

The mechanism of combination is complex and cannot be easily predicted from the activity of each compound in the mixture; therefore, understanding the synergistic mechanism is vital in selecting combinations. Moreover, the combination mechanism depends on several different variables, such as concentration, ratio, orientation, reaction medium, nature of radical initiators, interfering substances, and the microenvironment (21). For example, an inappropriate concentration of bioactive compounds in a mixture may lower its biological effects. Sulfated polysaccharides extracted from *Eisenia arborea* and *Solieria filiform* showed *in vitro* antiviral activity on the measles virus and low cytotoxicity at inhibitory

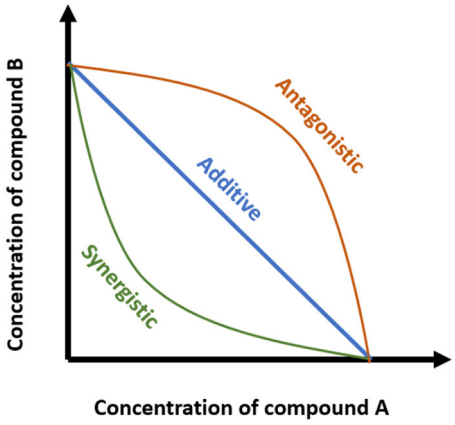


FIGURE 2
Isobole method showing different types of combination effects (synergistic, antagonistic, and additive). Axes represent the concentration of the individual compounds, and the points represent the combination of concentrations of the two compounds required to reach a particular fixed effect.

concentrations. When the synergistic effect of the two compounds was studied, the result showed that the synergistic effect of the combination was observed at low concentrations of extracts (96% inhibition with 0.0274 μg/mL and 0.011 μg/mL of *E. arborea* and *S. filiformis* sulfated polysaccharides, respectively.) in comparison to the higher individual extract effects (50% inhibition with 0.275 and 0.985 μg/mL of *E. arborea* and *S. filiformis*, respectively). Conversely, results showed an antagonism effect at high concentrations of extracts (22). Alongside the concentration, the proportion or ratio of individual compounds also plays an essential role in the synergistic activity of the mixture. Different ratios of bioactive compounds in combination can affect the synergistic interaction. For instance, Todorova et al. based on the CI calculation reported that the taxifolin/fucoidan combination at a ratio of 1:3 (CI 0.55) has a greater synergistic effect than the observed lower effect at a 3:1 ratio (CI 0.80) (23). Overall, the mechanism of synergy can be determined by increasing the bioavailability, enhancing the antioxidant activity, inhibiting lipid peroxidation, neutralization of adverse effects, and developing multi-target effects in different positions of a similar signaling cascade (Figure 3) (21).

## Method

A literature search was conducted using the PubMed, Scopus, and Google Scholar databases, and the search terms "Marine Bioactive Compounds," "Antioxidant Activity," "Anti-Inflammatory Activity," "Anticancer Activity," "Synergistic Effect," and "Additive Effect" were used. In this review, we focused on relevant articles and reviews on the health benefits of the combination of bioactive compounds and extracts from marine organisms for the prevention or treatment of chronic non-communicable diseases from 2000 to 2022. Studies on combinations of marine bioactive compounds with synthetic drugs and drug therapy are excluded. The results are presented and discussed in the following sections.

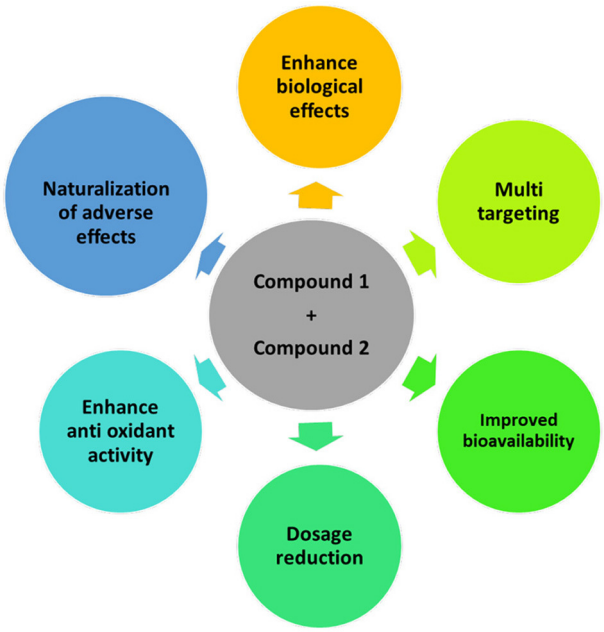


FIGURE 3
Mechanism of synergism—improvement in antioxidant activity, bioavailability, and biological effects of compounds; reduction of the dosage of individual compounds; act on new targets or multiple targets, or multiple pathways; naturalization of the adverse effect of individual compounds.

## Benefits of the combination of marine compounds

### Effects of bioactive compounds' interactions on anti-obesity activity

The prevalence of obesity has increased worldwide in the past few decades and is ascribed predominantly to poor eating habits. Many epidemiological studies show a decreased prevalence of obesity-related illnesses in those who consume seafood-rich diets, suggesting that marine foods have a positive effect against obesity. This disorder is not only limited to being overweight but can include comorbidities such as hyperlipidemia, cardiovascular diseases, type 2 diabetes, and non-alcoholic fatty liver (NAFLD). Many compounds have been extracted and tested on anti-obesity effects from marine organisms, mostly from algae. Additionally, the anti-obesity properties of lipids obtained from marine sources are well known (Table 1). Developing functional foods from marine bioactive compounds could be an opportunity for the food industry to prevent and/or treat obesity (32, 33).

Several studies have shown that the combination of marine bioactive compounds with anti-obesity activity has advantageous over individual compound via different additive or synergistic mechanisms (Table 4). There are several approaches to treat or prevent obesity including restricting glucose absorption, boosting body fat mobilization, increasing energy expenditure, reducing adipose tissue development, and blocking fat absorption (33). Therefore, one combinatorial mechanism is to target multiple pathways to enhance the response. In a study, Maeda and colleagues found that combining a bioactive compound with anti-obesity activities (fucoxanthin) and marine oil has an potential additive or beneficial anti-obesity effect. Fucoxanthin is a carotenoid found in edible brown seaweeds such as *Undaria pinnatifida* and *Hizikia fusiformis*. Fucoxanthin likely reduces the risk of obesity by lowering body fat in obese subjects (33). Fucoxanthin's mechanism of anti-obesity is currently ascribed to the up-regulation of mitochondrial uncoupling protein 1 (UCP1), which has a significant role in energy expenditure in white adipose tissue (34). Similar to fucoxanthin, fish oil can enhance energy expenditure by unregulated UCP1 expression in adipose tissue and significantly decrease weight gain and body fat (35). Fish oil also seems to stimulate lipid oxidation in healthy adults and limit triglyceride accumulation in adipose tissue through the regulation of fatty acid metabolism in the liver, and as a result, limit fat cell trophic growth (25, 26). The described activity from their combination is more effective for attenuating the weight gain of white adipose tissue than feeding with fucoxanthin alone in a diabetic/obese (KK-Ay) murine model. It has been suggested that the combination of fucoxanthin and fish oil can suppress the weight gain of white adipose tissue through multiple mechanisms. In other words, the individual compounds in the mixture might be able to attack different sites and/or different pathways associated with white adipose tissue maintenance (34). In another study, the authors further observed that the benefit of this combination could be due to another mechanism. For investigations depending on the oral administration of variables, the solubility of fucoxanthin is an essential factor to consider. The reason that fucoxanthin alone has lower anti-obesity activity compared to the mixture with fish oil could be due to the low solubility and oral bioavailability of the fucoxanthin. The authors suggested improving the oral bioavailability and absorption rate of fucoxanthin by first dissolving the carotenoid in fish oil or medium-chain triacylglycerols (MCT). As a result, Maeda et al. discerned that the anti-obesity activity of fucoxanthin with MCT was higher than fucoxanthin alone (36). The involvement of compounds that enhance the bioactive component solubility and stability is a significant type of synergism that is often undervalued (16).

In a similar study, lipids from *U. pinnatifida* (containing fucoxanthin) have anti-obesity activity both *in vivo* and *in vitro*, but due to low stability and bioavailability, Okada et al. (37) suggested developing a lipid delivery system in capsule form. The capsule was made of phospholipids derived from scallops (which also show anti-obesity activity). The results show that the combination of these bioactive lipids caused significant reductions in body weight and fat mass and has an additive effect compared to administering either lipid alone. The mechanism of reduction in the body weight might be due to the increases in the expression of UCP1 and UCP1 mRNA in epididymal fat tissue of diabetic/obese (KK-Ay) mice. The main reason behind the additive effect of the combination is that the scallop phospholipid increased the delivery and enhanced the stability and bioavailability of *U. pinnatifida* lipid in mice (38). Generally, in addition to pharmacodynamic synergy, several marine bioactive extracts may improve the solubility, absorption, distribution, or metabolism of active components with or without having particular pharmacological effects. The bioavailability of active components is increased as a result of these combined pharmacokinetic effects (16).

It is important to note that in the above-mentioned studies, the combined effects are not categorized as synergistic, however, but as "beneficial" or "additive" effects. Importantly, even the additive effect is not simply a summation of their constituents, and it requires

TABLE 1 List of compounds isolated from marine sources with potential anti-obesity activity.

| Compound name/class | Marine source | Experimental method | Mechanism of action | References |
|---|---|---|---|---|
| Fish oil | Fish | High-fat-fed rats | Unregulated UCP1 expression in BAT/Enhanced peroxisomal fatty acid oxidation | (24) |
| | | High-fat-fed rat & Clinical trial | Suppressing VLDL synthesis & hepatic lipogenesis | (25, 26) |
| | | High-fat-fed mice | Decrease in lipogenesis, & activated PPARα-mediated fat oxidation | (27) |
| Fucoidan/sulphated polysaccharide | Brown algae *Ecklonia cava* | 3T3-L1 cells<br>High-fat diet induced obese mice | Reduce white adipose tissue lipid accumulation and increasing energy expenditure | (28) |
| Fucosylated chondroitin sulfate/Glycosaminoglycan | *Acaudina molpadioides* | 3T3-L1 cells | Enhanced expression of Wnt/β-catenin related factors, as well as PPARγ and SREBP1 expression | (29) |
| Astaxanthin/Carotenoid | *Haematococc-us pluvialis* | Otsuka long evans tokushima fatty rat | Reduce visceral adipose tissue and ameliorate lipid profile | (30) |
| Siphonaxanthin/Carotenoid | Green algae *Codium cylindricum* | Diet induced obese mice | Decrease expression of lipogenesis-related genes and increase expression of energy expenditure-related genes in adipose tissue. | (31) |

Uncoupling protein 1 (UCP1); Brown adipose tissue (BAT); Very low-density lipoproteins (VLDL); Peroxisome proliferator-activated receptor α (PPARα); Peroxisome proliferator-activated receptor γ (PPARγ); Sterol regulatory element-binding protein 1 (SREBP1).

the use of assessment models such as isobole or CI for calculating the interactions.

Mohamadi et al. (39) used the synergy assessment CI method to describe the type of combination effect occurring in their lipid accumulation study. Obesity is linked to an increased risk of NAFLD. The pathogenesis of NAFLD is due to several pathological events, such as insulin resistance, oxidative stress, apoptosis, and inflammation which enhance the fat accumulation in the liver. Therefore, using a combination of bioactive compounds targeting more than one pathological event may have higher efficacy for NAFLD therapy or prevention. The combination of polyunsaturated fatty acids (PUFAs) of fish oil and chicoric acid, consistent with this theory, reduced lipid accumulation in hepatic cells. Chicoric acid, a phenolic compound extracted from chicory leaves, has anti-oxidative and anti-inflammatory effects (40). PUFAs can suppress lipogenesis by targeting sterol regulatory element-binding protein-1 (SREBP-1), which is the main transcriptional regulator of lipid metabolism (41). As a result, the combination can have synergistic effects for NAFLD, associated with lipid accumulation and lipotoxicity. Based on Mohamedi et al. results, PUFAs and chicoric acid synergistically reduce lipid aggregation as observed by decreased oil red O staining and triglyceride levels in palmitate-induced hepatic HepG2 cells. The molecular mechanism of the synergy effect is the down-regulation of activated protein kinase (AMPK)-mediated (SREBP)-1/fatty acid synthase (FAS) and up-regulation of AMPK-mediated peroxisome proliferator-activated receptor α (PPARα)/uncoupling protein 2 (UCP2) signaling pathways (39).

Another additive or synergy mechanism could be considered to develop novel bioactive combinations is lowering the effective dose of agents to limit toxicity. Guo et al. (42) proved that the adverse effect of bioactive compounds, such as cytotoxicity and hemolytic activity, can be neutralized by using the desired bioactive compound in combination. Sea cucumber saponins (SCS) and eicosapentaenoic acid-enriched phospholipids (EPA-PL) are extracted from sea cucumber *Pearsonothuria graeffei* and *Cucumaria frondosa,* respectively. These two bioactive compounds could suppress lipogenesis, increase fatty acid oxidation, and lower lipid accumulation. However, the eicosapentaenoic acid is easily oxidized, and sea cucumber saponin has cytotoxic and hemolytic activities, which is recommended to use at a lower concentration. Guo et al. evaluated the synergistic impact of the combination at the ratio of 1:1 against orotic acid-induced NAFLD to minimize the side effects. According to their findings, the combination could significantly increase the expression of genes related to fatty acid β-oxidation, including peroxisome proliferator-activated receptor (PPARα), while significantly decreasing mRNA expression of genes involved in fatty acid biosynthesis, including fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), glucose-6-phosphate dehydrogenase (G6PDH), and malic enzyme. They made the conclusion that combining sea cucumber extracts had more protective benefits on NAFLD than using individual compounds alone (42). Although the authors described the interaction of the bioactive compounds as synergy, no proper synergistic assessment model has been used; therefore, the interpretation of this study may be questionable.

In conclusion, the solubility and stability of anti-obesity compounds of marine origin are the main drawbacks (43). Therefore, formulating combinations to address these concerns may be a useful strategy. Obesity is a complex, multifactorial disorder that can be treated or prevented by targeting multiple pathways; therefore, designing combinations that attack several molecular targets could be another advantageous strategy.

## Effects of bioactive compounds' interactions on anti-inflammatory and antioxidant activities

Inflammation is a physiological response to foreign organisms or tissue injuries. During the inflammatory response, several

inflammatory cells, including macrophages, neutrophils, and lymphocytes, are activated. As a result, these cells release chemical mediators such as vasoactive amines, peptides, eicosanoids, acute-phase proteins, and cytokines (Tumor Necrosis Factor (TNF)-α, Interferon-gamma (IFNγ), nitric oxide (NO), prostaglandin E2 (PGE2) Interleukin (IL)-6 and IL-1β, IL-2, IL-8, IL-12, IL-17) to mediate the inflammatory response (44). In addition, oxidative stress can cause inflammation by producing reactive oxygen species (ROS). Excessive ROS production has been reported to trigger the inflammatory process resulting in the synthesis and secretion of pro-inflammatory cytokines through the activation of nuclear factor-kappa B/active protein-1 (NF-κB/AP-1) complex (45). If the inflammation continues for extended periods without resolution, chronic inflammation may eventually result in the development of other diseases, such as chronic asthma, rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, psoriasis, and cancer (3). Therefore, it is vital to treat and minimize long-term, unresolved inflammation with the use of anti-inflammatory and antioxidant agents. Although a few synthetic anti-inflammatory medicines exist, with continuous use, they all seem to have adverse physiological impacts. Lately, there has been an increasingly positive trend in the intake of antioxidants or anti-inflammatory products made from natural bioactive components (45). A large number of natural products have been extracted from marine organisms with anti-inflammatory and antioxidant properties, such as carotenoids, fucoidans, phlorotannins, sesquiterpenoids, diterpenes, steroids, polysaccharides, alkaloids, proteins, PUFAs, and other promising bioactive compounds for further research (Table 2) (5, 58).

For example, fish oil has well-recognized anti-inflammatory properties due to the presence of omega-3 PUFAs, especially docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). However, one of the disadvantages is the sensitivity of n-3 PUFAs to oxidation (59). Lipid hydroperoxides are formed during digestion as a result of the oxidation of fatty acids. The formation of lipid oxidation products during digestion can be inhibited by using natural antioxidants in combination with a lipid co-constituent (60). Alternatively, carotenoids (such as astaxanthin) are lipid-soluble natural pigments in seaweed that have antioxidant activity due to their ability to quench singlet oxygen and scavenge free radicals. Accordingly, the adverse effects of oxidative sensitivity of bioactive compounds or extracts may be reduced by combining them with natural antioxidants, promoting additive or synergistic effects. Several *in vivo* and *in vitro* studies have been published to date that document the advantages of combining marine-derived antioxidant astaxanthin with fish oil and which conclude that this combination strategy is successful in enhancing the stability and efficacy of fish oil (61–63).

Otton et al. examined the effect of daily ingestion of fish oil/astaxanthin in combination and as individual compounds on oxidative stress parameters and the functional properties of lymphocytes isolated from Wistar rat's lymph nodes. The effects have been reported as reduced intracellular calcium concentration, reduced T- and B-lymphocyte proliferative capacity, enhanced enzymatic antioxidant capacity, and decreased superoxide anion, hydrogen peroxide, and NO• production. Therefore, the combination of astaxanthin and fish oil may have a slightly beneficial effect in preventing oxidative stress induced by PUFAs (62). This combination has also been investigated *in vivo* by Barros et al. (61) and leads to a similar conclusion based on examining the effect on oxidative stress and functional indexes of rat-isolated neutrophils. The mixture of fish oil/astaxanthin induced hypolipidemic/hypocholesterolemic effects in plasma and improved the phagocytic activity of activated neutrophils compared to the added astaxanthin or fish oil alone. The combination enhances the immune response by improving the glutathione-based redox balance in rat plasma and neutrophils. However, the study concluded that the effects of this combination were summative rather than synergistic. These studies have shown that the combined effect might not serve to improve the efficacy of a given active compound but rather act to reduce the adverse effects that the active agent may cause (16).

The use of isolated bioactive components or extracts at concentrations greater than their physiological ones is a concern in most *in vitro* investigations. However, *in vivo*, bioactive compounds are found in plasma or tissues at lower levels. Another advantage of using marine antioxidant compounds in combination is that mixtures can significantly decrease the dose of each antioxidant needed to achieve an effect (16). Natural antioxidants alone usually are effective at considerably higher concentrations. Using appropriate combinations of different natural/synthetic antioxidants, which can function synergistically, can solve this issue. The mixture of antioxidants might increase the antioxidant activity and reduce the dose of a single natural antioxidant in higher concentrations (16). Todorova et al. evaluated the interactions between the natural antioxidants taxifolin (a flavonoid) and fucoidan. The ABTS radical cation decolorization assay was used to determine the antioxidant capacity of pure taxifolin, fucoidan and their combinations. The study aimed to minimize potential adverse effects from the overuse of a single antioxidant and to evaluate the synergistic activity of combinations by the use of this chemical method (23) (The advantages and disadvantages of using chemical-based methods are discussed in more detail in the discussion section). In this decolorization study, only one chemical method has been used to assess the antioxidant effect. The antioxidant activity involves a complex process that is regulated by numerous mechanisms. When assessing the antioxidant activity of individual substances or in combination, more than one test is required due to the complexity of the measured antioxidant's capacities. This fact is exemplified by Saw et al. who, besides utilizing only the chemical method, also confirmed the antioxidant activity of the tested combination by measuring glutathione levels and a total antioxidant power *in vitro* cell assay (63).

Several studies have investigated the interactions between marine extracts in terms of synergistic or additive anti-inflammatory activity. For example, phlorotannins of *Ecklonia cava* and sulfated polysaccharides and crude extracts of *Sargassum horneri* have anti-inflammatory effects under *in vitro* and *in vivo* conditions (64–66). Sanjeewa et al. suggest that the combination of *E. cava* and *S. horneri* extracts have higher anti-inflammatory activity than *E. cava* or *S. horneri* extracts alone. The 8:2 combination of *E. cava : S. horneri* extracts significantly inhibited the inflammatory mediators (iNOS, COX-2, IL-1β, IL-6, and TNF-α) in lipopolysaccharides-activated RAW 264.7 macrophage cells compared to the single extracts (66). In other words, this interaction can boost the mixture's activity compared to individual compounds. They concluded that the synergistic effect of the combination could be due to the different

TABLE 2 List of selected compounds isolated from marine sources with potential anti-inflammatory and anti-oxidant activity.

| Compound name/class | Marine source | Experimental method | Mechanism of action | References |
|---|---|---|---|---|
| Lemnalol/Sesquiterpenoid | *Lemnalia cervicorni* | RAW264.7 macrophages & Carrageenan-activated rat paws | Inhibition of iNOS and COX-2 expression | (46) |
| Clovane compound 1/Sesquiterpenoid | *Rumphella antipathies* | FMLP/CB-activated human neutrophils | Inhibition of superoxide anions generation | (47) |
| 8-hydroxybriaranes/ Diterpenoids | Sea whip *Junceella fragilis* | RAW264.7 macrophages | Inhibition of COX-2 and iNOS release | (48) |
| Excavatolide B/Diterpene | *Briareum excavatum* | Carrageenan-activated rat paws | Inhibition of iNOS expression | (49) |
| Lobocrasol A/Diterpene | *Lobophytum crassum* | HepG2 cells | NF-κB activation | (50) |
| Solomonsterol A/Steroid (sterol) | *Theonella swinhoei* | Arthritis mice model | Reduction in arthritic score | (51) |
| Ergosta-7,22-dien-3-ol/Steroid (sterol) | *Marthasterias glacialis* | RAW264.7 macrophages | Inhibition of iNOS protein level | (52) |
| Steroid compound 5/Steroid (sterol) | *Astropecten polyacanthus* | Mice bone marrow-derived dendritic cells | IL-12 p40, IL-6, and TNF-α production | (53) |
| Sulfated polysaccharides/Polysaccharide | *Porphyra haitanensis* | Tropomyosin-induced mouse allergy model | Decrease in IgE level | (54) |
| Polysaccharides | *Digenea simplex* | Carrageenan-activated mouse paws | Reduction of edema volume | (52) |
| Indole-4-carboxaldehyde/Alkaloid | Brown seaweed *Sargassum thunbergii* | HepG2 cells | Limiting expression of pro-inflammatory genes | (55) |
| Lectin/Protein | *Caulerpa cupressoides* | Zymozan-activated rats | Reduction of leukocyte counts and myeloperoxidase activity | (56) |
| Hexadecanoic acid/Fatty acid 1-(15- methyl-1-oxohexadecyl)-pyrrolidine / Pyrrolidine | *Octopus vulgaris* ink extracts | RAW264.7 macrophages | Increase secretion of main cytokines from JAK-STAT, PI3K-Akt, and IL-17 pathways | (57) |

Inducible nitric oxide synthase (iNOS); Cyclooxygenase-2 (COX-2); Nuclear factor-κB (NF-κB); Interleukin (IL)-12 IL-12 p40, IL-6, IL-17; Tumor necrosis factor α (TNFα); Immunoglobulin E (IgE); Janus kinase (JAK)-signal transducer and activator of transcription (STAT); Phosphoinositide-3-kinase–protein kinase B/Akt (PI3K-PKB/Akt).

bioactive properties of active compounds present in the extracts. The studies that examine the interaction between marine extracts provide more insight into the combined effect of several kinds of bioactive compounds. However, they cannot establish the synergy of individual compounds. It is suggested to identify the active compounds in the extracts, and single compounds are tested solely and in combination.

Correspondingly, marine extracts with anti-inflammatory or antioxidant activity could also be a source of marine-based prebiotics to combine with probiotics for developing more effective dietary supplements. Such symbiotic combinations may have a greater effect on improving intestinal health and avoiding inflammation of the intestines. *Gracilaria coronopifolia* seaweed extract, as a prebiotic, was combined with several probiotics (*Lactobacili* and *Bifidobacteria*), and the synergistic efficacy of the mixture was evaluated by Li et al. using the intestinal Caco-2 cell line model. Based on the result of the study, *the G. coronopifolia*/probiotic mixture inhibits ROS production under oxidative stress, reduces the damage of cells with oxidative stress, and inhibits the inflammatory response in cells. In addition, the combination can protect, maintain, and improve the function of intestinal cells by inhibiting the production of inflammatory factor substances (interleukin 8, IL-8) (67).

In general, inflammation and oxidative stress, which results in the production of chemical mediators and reactive oxygen species (free radicals), are linked to several chronic illnesses, including cancer, cardiovascular disease, and type 2 diabetes mellitus; therefore, designing combinations including bioactive compounds with both anti-inflammatory and antioxidant activities can be a promising scheme in chronic disease treatment and prevention.

## Effects of bioactive compounds' interactions on anticarcinogenic activity

Cancer development increases with age, and the beginning of cancer is often a slow process. Consuming functional food with anti-cancer properties is considered a chemo-preventative approach to offset or reduce the chance of cancer development (68). Chemo-preventative nutraceuticals inhibit the development of a healthy cell into a cancer cell by inducing antioxidant and anti-inflammatory activity, inactivation of phase I enzymes, and induction of phase II enzymes. Chemotherapeutic nutraceuticals, on the other hand, target cancer cells directly by inhibiting tumor growth (inhibiting cell proliferation, inducing cell differentiation, and apoptosis) or inhibiting tumor spread (inhibiting tumor invasion, anti-metastasis) (6).

TABLE 3 List of selected marine bioactive compounds with anti-cancer activity.

| Compound name/class | Marine source | Experimental method | Mechanism of action | References |
|---|---|---|---|---|
| Fucoidan/Polysaccharides Laminarin/Polysaccharides | *Sargassum hemiphyllum* | Lung tissue from mice | Altered the expression patterns of inflammatory cytokines, reducing radiation pneumonitis and radiotherapy-induced lung fibrosis | (69) |
| | *Laminaria digitata* | Human colon cancer cells (HT-29) | Induces apoptosis & suppresses ErbB signaling pathway activation | (70) |
| Dieckol/Polyphenol | *Ecklonia cava* | HT1080 cells | Downregulates FAK signaling pathway mediated by ROS | (71) |
| Diphlorethohydroxycarmalol/ Phlorotannin | *Eisenia bicyclis* | HL60 cells | Induces apoptosis & reduces Bcl-2 expression & depletes mitochondrial membrane potential | (72) |
| HFGP/Glycoprotein | *Hizikia fusiformis* | HepG2 cells | Induces apoptosis & upregulates expressions of Fas, Fas-associated death domain protein, Bax, and Bad | (73) |
| | | SMMC-7721 cells | Induces apoptosis *via* ROS-mediated mitochondrial pathway | (74) |
| Fucoxanthin/Carotenoid | Brown algae | Carcinogenic murine azoxymethane/dextran sodium sulfate (AOM/DSS) mice model | Exhibit chemopreventive potential by downregulation of HSP70 genes | (75) |
| Pheophorbide a/Chlorophyll | *Grateloupia elliptica* | Glioblastoma cells (U87 MG) | Induces cytostatic activity | (76) |
| Clerosterol/Sterol | *Codium fragile* | | Induces apoptosis & increase of Bax & decrease of Bcl-2 expression & activates Caspase-3/Caspase-9 | (77) |
| Astropectenols/Sterol | *Astropecten polyacanthus* | HL-60 cell line | Induces caspase dependent apoptosis & regulates Bcl-2, Bax, PARP | (53) |
| Pleurocidin-Amide/Peptides | *Pleuronectes americanus* | A549 lung adenocarcinoma cells | Inhibit autophagy of A549 cells, and induce apoptosis | (78) |
| Aplysin/Terpenoids | *Aplysia kurodai* | A549 NSCLC cell line | Anti-neoplastic agent & induces intrinsic/extrinsic apoptosis | (79) |
| Mandelalide A-D/Polyketides | *Lissoclinum mandelai* | Proliferating tumor cells | Induces intrinsic apoptosis & inhibits mammalian ATP synthase complex V | (80) |
| N-(2-ozoazepan-3-yl)-pyrrolidine-2-carboxamide/Pyrrolidine | *Octopus vulgaris* ink extracts | Human cancer cell lines (22Rv1, HeLa, A549) | Anti-proliferative, early-apoptosis induction, reactive species modulation, and nuclei disruption in 22Rv1 cells | (81) |

Focal adhesion kinase (FAK); Reactive oxygen species (ROS); Heat shock protein 70 (HSP70).

Bioactive compounds such as polyphenols, polysaccharides, alkaloids, peptides, and terpenoids have been isolated from marine organisms and elicit anti-cancer activity *in vitro* and *in vivo* (Table 3). The main source of these isolated bioactive compounds is marine microbiota, including microalgae, fungi, seaweeds, mangroves, bacteria, cyanobacteria, and actinobacteria. The mechanism of action is mainly through inhibiting tumor growth *via* augmenting and supporting apoptosis, necrosis, and lysis of the tumor cells (82).

Marine bioactive compounds or extracts with antioxidant activity could be a decent candidate to prevent cancer development since many chronic diseases, including cancer, are caused by oxidative stress (63). Astaxanthin has antioxidant activity and inhibits oxidative damage, which could explain its chemo-preventative activity. Accordingly, Saw et al. suggested that a lower concentration of astaxanthin has potent antioxidant activity in conjunction with PUFAs (DHA or EPA) and demonstrates synergistic antioxidant effects. Inducing the nuclear factor erythroid 2-related factor 2/ antioxidant response elements (Nrf2/ARE) pathway in an *in vitro* HepG2-C8-ARE-luciferase cell line, beneficial synergism is aligned with the pathway which has the main role in the induction of antioxidant genes that protect against oxidative damage (63). As a result, the authors have suggested that this combination is a promising cancer chemoprevention strategy. In this study, the synergism has been confirmed by using CI method.

The mechanism of synergy in Saw et al. (63) study is to reduce the concentration of bioactive compounds in combination to produce better efficacy with less toxicity than individual compounds. However, cancer is a multi-targetable disease, and the combination of compounds that address many targets simultaneously can be a better approach to treat various types of cancer. Designing marine-based combinations that promote cell death, inhibit cell proliferation and invasion, and boost the immune system simultaneously are future possibilities. Moreover, high biological activity and the wide availability of these marine compounds are advantages that should be considered (83). Most studies focus on the synergistic effect of marine compounds in combination with existing anti-cancer drugs and enhancing drug therapy

TABLE 4 List of combination of marine bioactive compounds.

| Compound name | Experimental method | Mechanism of action | Combination effect | Combination effects assessment | References |
|---|---|---|---|---|---|
| **Anti-obesity activity** | | | | | |
| Fucoxanthin & fish oil | Diabetes/obesity mouse model (KK-Ay). | Plural mechanisms | Beneficial effect on the attenuation of WAT weight gain | Potential additive or beneficial | (34) |
| Fucoxanthin & MCT | Diabetes/obesity mouse model (KK-Ay). | Up-regulation of mitochondrial UCP1 | Enhanced anti-obesity effects | Enhancement or beneficial | (36) |
| | | | Increasing the bioavailability of fucoxanthin | | |
| Fucoxanthin & phospholipids of scallop | KK-Ay mice | Increases in the expression of UCP1 and UCP1 mRNA | Significant reductions in body weight and fat mass | Potential additive effect | (38) |
| Fish oils & chicoric acid | Hepatic HepG2 cells | Down-regulation of AMPK-mediated SREBP-1/FAS and up-regulation of AMPK-mediated PPARα/UCP2 signaling pathways | Synergistically reduce lipid aggregation | Synergistic (CI) | (39) |
| Sea cucumber saponins & eicosapentaenoic acid-enriched phospholipids | Orotic acid-induced NAFLD rats | Increase expression of PPARα. Decreasing mRNA expression of FAS, ACC, G6PDH, malic enzyme | Synergistic effect on lipogenesis inhibition and β-oxidation enhancement | Potential synergistic (No synergy analysis) | (42) |
| **Anti-inflammatory and Antioxidant activity** | | | | | |
| Fish oil (PUFAs) & astaxanthin | Wistar rats Lymphocytes | Reduced proliferative capacity of T- and B-lymphocytes, Minimize superoxide anion, hydrogen peroxide, and NO● production | Preventing Lipid hydroperoxide | Enhancement or beneficial | (62) |
| | | | Preventing oxidative stress caused by PUFAs | | |
| | | | Stimulating the immuno-modulatory effects of fish oil | | |
| Fish oil (PUFAs) & astaxanthin | Wistar rats neutrophils | Improving the glutathione-based redox balance | Induced hypolipidemic/hypocholesterolemic effects in plasma | Enhancement or beneficial | (61) |
| | | | Improved the phagocytic activity of activated neutrophils | | |
| Astaxanthin & omega-3 fatty acids | HepG2-C8-A cell line | Inducing the Nrf2/ARE pathway | Synergistic antioxidant Protect against oxidative stress | Synergistic (CI) | (63) |
| Taxifolin (flavonoid) & fucoidan | Chemical method (ABTS) | | Decrease the dose needed of each bioactive compound | Synergistic | (23) |
| | | | | (Isobole method) | |
| *Gracilaria coronopifolia* seaweed extract & probiotic | Caco-2 cell line | | Improve intestinal health | Enhancement or beneficial | (67) |

*(Continued)*





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TABLE 4 (Continued)

| Compound name | Experimental method | Mechanism of action | Combination effect | Combination effects assessment | References |
|---|---|---|---|---|---|
| | | | Avoid inflammation of the intestines | | |
| Phlorotannins of *E. cava* & sulfated polysaccharides and crude extracts of *S. horneri* | RAW 264.7 macrophage cells | Blocking NF-κB and MAPK pathways | Synergistic anti-inflammatory effects | Potential synergistic (No synergy analysis) | (87) |
| **Chemopreventive activity** | | | | | |
| Astaxanthin & omega-3 fatty acids | HepG2-C8- A cell line | Inducing the Nrf2/ARE pathway | Synergistic antioxidant Protect against oxidative stress | Synergistic (CI) | (63) |

NF-E2-related factor 2 (Nrf2); Antioxidant response element (ARE); Mitogen-activated protein kinases (MAPKs); Focal adhesion kinase (FAK); Acetyl CoA carboxylase (ACC); Glucose-6-phosphate dehydrogenase (G6PD).

(84–86). Investigating the synergistic interaction between naturally occurring bioactive compounds or extracts with anti-cancer activity is highly recommended.

## Discussion and future perspective

Several combinations of marine compounds and extracts have been studied, and their interaction mechanism has been discussed in this review. Of all mechanisms, enhancing the bioavailability and reducing adverse effects of active compounds are the most frequently reported. However, in the case of chronic non-communicable diseases that involve different pathogenesis pathways, the ideal combination may be applying compounds with non-overlapping activities to provide either additive or synergistic response on multiple molecular targets. Inflammation and oxidative stress are the two main aspects of chronic diseases. Therefore, combinations that target pathways in relation to these two aspects are considered useful approaches for therapy or prevention. Future research should concentrate on finding such combinations.

The diversity of the marine compounds or extracts examined in combinations should be increased. Most studies have focused on fish oil and carotenoids (mostly fucoidan, astaxanthin, and fucoxanthin) to develop novel combinations (Table 4). Future research should investigate the combinatorial effect of other promising marine bioactive compounds. In recent years, attention has been drawn to a greater extent to marine proteins and peptides. These bioactive compounds have a range of biological properties, including immunomodulating, neuroprotective, anti-diabetic, anti-cancer, antiviral, and anti-oxidative aspects (88, 89). Despite their significant bioactivity, there are several problems to use these compounds in functional foods and nutraceuticals, such as poor solubility, unfavorable taste, low bioavailability, and stability (1). Applying peptide or protein in combination with other bioactive compounds or extracts may be a solution to improving their water solubility, increasing their bioavailability, improving sensory properties, increasing their food matrix compatibility, improving their stability in the gastrointestinal tract, and protecting bioactive components from unfavorable food matrix environment.

Moreover, less attention has been paid to design combinations with anti-cancer activity. To our knowledge, unfortunately, no study has yet been published which investigates the synergistic effect of naturally occurring marine bioactive or marine extracts as chemotherapeutic or chemopreventive functional foods. This research gap should be filled by encouraging more studies to develop novel combinations with anti-cancer activities. The current repository of studies on the combination of phytochemicals with defined anti-cancer activity (which show promising results both *in vitro* and *in vivo*) may serve as guides to future investigations (12–15).

In addition, the combinatorial effects of marine bioactive components in the human body are currently lacking as a source of information. Most marine combination studies have been carried out as *in vitro* (cell culture assays, for example) or *in vivo* (animal) models; therefore offer limited information regarding the bioavailability and biotransformation of the components in the human body after consumption.

Synergistic, additive, or antagonistic factors of the interactions in combinations must be determined to avoid misconceptions in developed synergy. Some studies do not categorize the combinational interaction or distinguish between synergistic and additive effects. The synergistic and additive effects are two different interactions that should not be confused. Advantageously, the combined effect should be clearly and accurately defined. Consequently, to identify the combined effect and to facilitate the comparison of results between relevant research, it is recommended to use analytical methods such as Isobole analysis or CI (16).

Developing novel natural bioactive combinations is likely a complicated and time-consuming project. Several factors should be considered before planning the investigation. The first factor to consider is the knowledge of the mechanism of action of selected individual compounds and their combinations. Since the mode of action of a combination could be different from that of the same compounds acting individually, understanding the mechanism of synergistic effects of combinations is important for the future development of new marine bioactive combinations and functional food (16). For example, sulphated polysaccharides extracted from the seaweeds *Ulva clathrate* and *Cladosiphon okamuranus* (fucoidan) have antiviral properties against paramyxovirus infection. However, the combination has no clear synergistic advantage, and the *U. clathrate* extract antagonizes the effect of fucoidan on the viral attachment/entry in the mixture, probably because both act on the same target (90).

Selecting an appropriate model for testing the combined effect is the main step to obtaining relevant data for understanding the combination effects. The cell-free chemical model is not a convenient method to test the combinational effect. It mainly relies on the solvent (in which the compounds are dissolved for the experiment), the concentration and volume ratio of the individual compounds, and the estimation method for testing the combination effect. As a result, the data concluded from the chemical method may not be relevant to biological systems (e.g., cell culture, animals, and humans). Consequently, this may lead to incorrect conclusions and mislead the study (91). Improving overall conditions for the experimental context, *in vivo* animal models replicate the physiology of an entire organism. However, this type of model has several drawbacks. Inconsistency in model responses due to species variations, difficulties in interpreting results to humans, dealing with ethical issues, and the effort required to maintain an animal facility are prominent issues. Therefore, the advanced cell culture method, such as co-culture, can be an alternative method to animal testing. In this model, cell culture is conducted by using one or more cell lines in (co-) culture under two-dimensional (2D) settings. Co-culture can more closely resemble an *in vivo* model, allowing for the efficient and reproducible investigation of molecular mechanisms of combinations and uncovering multi-target mechanisms (92). For instance, co-culture models of immune and intestinal cells can be developed to study the anti-inflammatory/antioxidant activities of the food combination in relation to the compounds' absorbance and bioavailability after digestion in a cell model (93, 94). Due to technological advancements, three-dimensional (3D) cell culture has also been developed to imitate the complex composition of human tissue and organs. This model can be used for in-depth investigations of molecular mechanisms of combinational effects (95).

## Conclusion

Marine functional foods and combination therapy have increasingly been recognized and studied as viable and successful approaches for treating or preventing chronic non-communicable diseases. Several combinations of marine-based bioactive compounds or extracts have been proven to possess synergistic or enhanced therapeutic effects, notably antioxidant, anti-inflammatory, anti-obesity, and chemo-preventative activities. The most often stated benefits of combinations involve increasing the bioavailability of compounds and minimizing their adverse side effects. The best combinatorial approach, however, should focus on targeting multiple pathways of chronic non-communicable diseases. In addition, there are many marine bioactive compounds with known biological properties, but only a few have been tested in combination. The limited research in the field of marine natural combinations provides an incredible opportunity for researchers to develop novel functional foods exhibiting higher efficacy within the context of disease prevention and treatment. However, for developing novel marine bioactive combinations, several factors should be considered to ensure confidence in the applicability of the tested combinations.

## Author contributions

BE performed the literature search and wrote the original manuscript. SB edited the original manuscript. JLi, TY, and BZ edited the final version of the manuscript. JLu conceived the idea and edited the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The 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.

## Publisher's note

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# Effect of dietary phenylalanine on growth performance and intestinal health of triploid rainbow trout (*Oncorhynchus mykiss*) in low fishmeal diets

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This study aimed to investigate the effects of phenylalanine on the growth, digestive capacity, antioxidant capability, and intestinal health of triploid rainbow trout (*Oncorhynchus mykiss*) fed a low fish meal diet (15%). Five isonitrogenous and isoenergetic diets with different dietary phenylalanine levels (1.82, 2.03, 2.29, 2.64, and 3.01%) were fed to triplicate groups of 20 fish (initial mean body weight of 36.76 ± 3.13 g). The weight gain rate and specific growth rate were significantly lower ($p < 0.05$) in the 3.01% group. The trypsin activity in the 2.03% group was significantly higher than that in the control group ($p < 0.05$). Amylase activity peaked in the 2.64% treatment group. Serum superoxide dismutase, catalase, and lysozyme had the highest values in the 2.03% treatment group. Liver superoxide dismutase and catalase reached their maximum values in the 2.03% treatment group, and lysozyme had the highest value in the 2.29% treatment group. Malondialdehyde levels in both the liver and serum were at their lowest in the 2.29% treatment group. Interleukin factors IL-1β and IL-6 both reached a minimum in the 2.03% group and were significantly lower than in the control group, while IL-10 reached a maximum in the 2.03% group ($p < 0.05$). The tight junction protein-related genes occludin, claudin-1, and ZO-1 all attained their highest levels in the 2.03% treatment group and were significantly higher compared to the control group ($p < 0.05$). The intestinal villi length and muscle layer thickness were also improved in the 2.03% group ($p < 0.05$). In conclusion, dietary phenylalanine effectively improved the growth, digestion, absorption capacity, antioxidant capacity, and intestinal health of *O. mykiss*. Using a quadratic curve model analysis based on WGR, the dietary phenylalanine requirement of triploid *O. mykiss* fed a low fish meal diet (15%) was 2.13%.

KEYWORDS

**phenylalanine, requirement, triploid *Oncorhynchus mykiss*, intestinal health, low fish meal diet**

## Introduction

Fish meal is the main protein source for fish feed (1). Due to limited fish meal resources and rising prices, the use of fish meal in fish feed has always been reduced by replacing animal protein sources with plant protein sources in the past decades (2). However, there are some problems, such as nutrient deficiency and indigestion after eating, which hinder the development of fish meal replacement. Fish farming depends on whether the nutritional balance of the feed received by the fish is up to standard (3). Lack of nutrients, especially essential amino acids, can have a serious impact on the growth and health of fish (4). In plant-based feed formulations, critical amino acids including methionine, lysine, and threonine are frequently restricted by amino acids that are frequently added as feed supplements. Plant protein sources may be low in other essential amino acids compared with fish meal (5). Furthermore, fish have lower availability and utilization of plant protein, thereby affecting the availability of essential amino acids in fish. Current practice in the formulation of fish diets is to add methionine, lysine, and threonine to prevent deficiency of essential amino acids (6). Levels of amino acids in the standard were determined and optimized for purified, semi-purified, or fishmeal-based diets and may be insufficient for fish fed a plant-based diet.

Phenylalanine is an aromatic amino acid, which is one of the essential amino acids for fish (7). It is converted to tyrosine in the liver and kidneys, which in turn is a precursor to epinephrine and norepinephrine, thyroid hormone, triiodothyronine, and thyroxine (8). They participate in the functional role of brain chemistry by crossing the blood–brain barrier (9). Tyrosine is also known as a semi-essential amino acid due to the need for additional phenylalanine supplementation to meet the growth and metabolic requirements of fish production when tyrosine levels are insufficient. Aromatic amino acids have an irreplaceable role throughout growth, metabolism, and protein synthesis. It has been determined that a deficiency of phenylalanine in fish will result in decreased feed utilization, reduced antioxidant performance, and impaired growth performance (10, 11). The improvement of phenylalanine in the growth performance of fish may be related to its ability to improve the feed utilization of fish. In previous studies, it was found that the feed utilization rate of aquatic animals such as Indian major carp (*Cirrhinus mrigala*) (12) and catfish *Heteropneustes fossilis* (13) increased with an increase in phenylalanine levels. Molecules like mTOR are able to integrate and regulate the relationship between various nutrients and growth signals in order to regulate the balance between the body's growth and proliferation rate and the intake of external nutrients. The expression of IGF-1 and mTOR in the hepatopancreas was significantly activated by the addition of phenylalanine to the diet of *Portunus trituberculatus* (14). After the upstream mTOR pathway is activated, the downstream S6K1 and 4EBP-1 genes will also show higher expression levels.

As an important digestive gland, the pancreas secretes a variety of enzymes that can digest protein, lipids, and so on. After the protease passes through the pancreatic duct, it forms trypsin under the action of enterokinase and further activates other proteases. Lipase hydrolyzes glycerides and phospholipids by cutting off lipid bonds (15). Phenylalanine can promote the secretion of protease and bicarbonate in the dog pancreas (16). However, studies in chickens showed that phenylalanine could not promote the secretion of amylase (17). There are few reports on the effects of phenylalanine on the growth and development of fish digestive organs. Only the digestive performance of Jian carp (*Cyprinus carpio* var. Jian) was improved after the phenylalanine supplement (18). Therefore, there may be a positive significance in studying the effect of phenylalanine on the digestive ability of trout.

To resist the damage caused by oxidation, fish also contain antioxidant enzymes, including superoxide dismutase (SOD) and catalase (CAT) (19). Phenylalanine is a precursor of tyrosine, which in turn is a precursor of dopamine and thyroxine. In cultured astrocytes, dopamine increased extracellular SOD protein expression and cell surface SOD activity (20). Thyroxine increased GPx activity and GSH levels in erythrocytes (21). These findings clarify the effects of the antioxidant properties given by phenylalanine to fish. In juvenile carp, it was found that a lack or excess of phenylalanine down-regulated CAT activity, while excess phenylalanine down-regulated SOD gene expression; CAT and SOD gene expression could be up-regulated only when added in appropriate amounts (22). In grass carp (*Ctenopharyngodon idella*), it was shown that 9.57 g/kg of dietary phenylalanine could reduce malondialdehyde (MDA) content in the gills (22).

The intestinal immune barrier in fish is mainly controlled by intestine-associated lymphoid tissues such as monocytes, lymphocytes, macrophages, and granulocytes (23). Nutrients, on the other hand, can modulate the immune system of the intestine by affecting the structural integrity of the intestine (24). In fish, phenylalanine is secreted to produce melanin (25). It was found that melanin can reduce the production of cytokines such as interleukin 1-beta (IL-1β) and interleukin-6 (IL-6) in the body. However, whether dietary phenylalanine has a similar effect on trout has not been reported yet, so whether there is a correlation between phenylalanine and these cytokines deserves further study.

According to the food and agriculture organization of the United Nations (FAO), the annual production of farmed salmon and trout exceeds 3 million tons, making it the third-largest aquaculture species in the world as of 2020 (26). Recently, trout farming in China has developed rapidly and has become one of the main coldwater fish farming species in China, with annual production already reaching 30,000 tons (27). Triploid *Oncorhynchus mykiss* has a faster growth rate, a lower feed coefficient, and a higher meat content than diploid, and it is now the main cultured species of coldwater fish in China (28). The main objective of this study was to investigate the effects of dietary phenylalanine levels on growth performance, intestinal digestive and immune enzyme activity, intestinal gene expression of inflammation and tight junction protein, and the antioxidant capacity of digestive organs of triploid *O. mykiss* fed a low fish meal diet. This will be essential as triploid *O. mykiss* feeds move toward precision formulation.

## Materials and methods

### Feed formulation and preparation

According to the nutritional needs of *O. mykiss*, fish meal and soybean meal were employed as the protein sources, soybean oil and fish oil were used as the sources of lipids, and dextrin was included as the carbohydrate sources. The basic feed with a

crude protein level of 41.01% and a crude lipid level of 11.76% was prepared as the control group [phenylalanine level of 1.82% (G1)]. To achieve 2.03% (G2), 2.29% (G3), 2.64% (G4), and 3.01% (G5) phenylalanine levels in the feed, 0.30, 0.60, 0.90, and 1.20% L-phenylalanine (Sigma, 99%) were added, respectively. The tyrosine content was 1.15% (G1), 0.93% (G2), 1.03% (G3), 0.97% (G4), and 1.14% (G5), respectively. Prepare the ingredients according to the formula, and then put them into the mixer and mix well. Ingredients were finely ground before mixing (<250 μm) and then blended with minerals and vitamins. After adding the lipid source, all ingredients were thoroughly mixed for 25 min. Distilled water was then added to achieve the right pellet consistency. The mixture was further homogenized, and a pelletizer (GYJ-250B, Dashiqiao Bao Feed Machinery Factory) was used to form 1-mm pellets. Pellets were dried until the moisture content decreased to about 10% in a ventilated oven at 60°C, and were then stored at −20°C for further use. The formula and nutritional level of the experimental feed are shown in Table 1, and the composition and amount of amino acids in the feed are shown in Table 2.

## Feeding trial

Triploid *O. mykiss* was purchased from Egremorin Industries (Benxi, China) and acclimated for 15 days. Control diets were fed throughout the acclimate period. Before the feeding experiment, a total of 300 fish with an initial average weight of (36.76 ± 3.13 g) were allocated to 15 tanks, with 20 healthy and uniform fish per replicate, and three replicates per treatment group. The experiment was carried out in an indoor aquarium with a controlled water circulation system. A feeding trial was conducted for 8 weeks, during which the fish were fed the test diets twice daily, at 9:00 a.m. and 4:00 p.m., until satiation.

Feeding condition: the water source was aerated tap water. Water temperature was maintained at 14 ± 0.5°C. The water dissolved oxygen concentration is >6.0 mg/L, $NO_2^-$-N < 0.02 mg/L, pH 6.8–7.1, and $NH_4^+$-N < 0.2 mg/L, respectively. Water quality parameters were measured using a YSI-556 multi-parameter water quality meter (YSI Inc., Yellow Springs, OH, USA). One-third of the water is changed every afternoon to ensure water clarity and sufficient dissolved oxygen.

## Sample collection

At the end of the experiment, fish were starved for 24 h to allow emptying of the digestive tract contents prior to sampling. All fish were weighed to calculate weight gain rate and other growth indicators [ME204E, Mettler-Toledo Technologies (China) Co.]. Nine fish were randomly selected from each treatment group and anesthetized with tricaine methanesulfonate MS-222 (75 mg/L). Blood samples were obtained from the tail vein, then centrifuged at 4,000 × *g* for 10 min at 4°C, and the supernatant was extracted as serum. The serum was stored at −20°C for subsequent serum biochemical assays. The mid-intestines of three fish were stored at −40°C for biochemical analyses. The intestines of the other three fish were removed and immediately frozen in liquid nitrogen and stored at −80°C at the end of sampling for subsequent gene expression assays. The other three fish intestines were stored in Bouin's solution for future histomorphological observation.

## Nutritional content

The experimental diets and fish were analyzed using an AOAC-based protocol (29). Moisture content was determined by drying the samples in an oven at 105°C until a constant weight was obtained. Crude protein (N × 6.25) was analyzed by measuring nitrogen using the Kjeldahl method (2300, FOSS, Sweden). Ash content was analyzed by carbonization at 300°C for 30 min, followed by incineration at 550°C for 4 h. Crude lipid was measured by the Soxhlet method (Extraction System-811, BUCHI, Switzerland).

## Amino acid determination

Before the start of amino acid determination of fish and feed, 40–50 mg (accurate to 0.1 mg) of the sample was weighed with an electronic analytical balance, and 10 ml of hydrochloric acid with a concentration of 6 mol/L was added. The ampoule was then heated by an alcoholic blowtorch and sealed immediately, and then placed in a constant temperature oven for 22 h of hydrolysis, setting the temperature at 110°C. After cooling, 10 ml of 6 mol/L sodium hydroxide solution was added to the alkali neutralization. Then the solution was poured into a 100 ml volumetric flask, fixed with 0.02 mol/L hydrochloric acid, and mixed well with the sample hydrolysis solution. The sample was filtered through a 0.2 μm filter membrane into the sample bottle before the machine, and then was determined by an automatic amino acid analyzer (L-8900, Hitachi, Japan).

## Biochemical analysis

Biochemical analysis assays were performed using commercially available kits according to the manufacturer's protocol (Nanjing Jiancheng Institute of Biological Engineering, Nanjing, China). CAT (A007-2-1) activity was determined by measuring the decrease in $H_2O_2$ concentration at 240 nm. The reaction mixture contained 50 mm of potassium phosphate buffer (pH 7.0) and 10.6 mM of freshly prepared $H_2O_2$. SOD) (A001-3-2) activity was measured spectrophotometrically using xanthine/xanthine oxidase as a source of superoxide radicals. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 0.1 mM xanthine, 0.013 mM cytochrome c, and 0.024 IU/ml xanthine oxidase. An activity unit was defined as the amount of enzyme required to produce 50% inhibition of the rate of reduction of ferrocyanic measured at 550 nm. The amount of lysozyme (LZM; A050-1-1) was measured by a turbidimetric assay. By destroying the β-1,4-glycosidic bond between n-acetyl acetylmuramic acid and n-acetyl glucosaccharide in the cell wall, the cell wall insoluble monosaccharide is decomposed into soluble glycopeptides, resulting in the rupture of the cell wall and the escape of the contents to make the bacteria dissolve. Lipid peroxidation was analyzed in MDA (A003-1-2) equivalents using

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TABLE 1 Experimental diet composition and nutrient levels (air-dry basis, g/kg).

| Items | Diets | | | | |
|---|---|---|---|---|---|
| | G1 (1.82%) | G2 (2.03%) | G3 (2.29%) | G4 (2.64%) | G5 (3.01%) |
| Fish meal[a] | 150.00 | 150.00 | 150.00 | 150.00 | 150.00 |
| Soybean meal[a] | 140.00 | 140.00 | 140.00 | 140.00 | 140.00 |
| Soybean oil[a] | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 |
| Fish oil[a] | 62.80 | 62.80 | 62.80 | 62.80 | 62.80 |
| Compound amino acids[b] | 136.80 | 136.80 | 136.80 | 136.80 | 136.80 |
| Dextrin | 200.00 | 200.00 | 200.00 | 200.00 | 200.00 |
| Gelatin | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 |
| Beer yeast | 50.00 | 50.00 | 50.00 | 50.00 | 50.00 |
| Soybean phospholipid | 20.00 | 20.00 | 20.00 | 20.00 | 20.00 |
| $Ca(H_2PO_4)_2$ | 10.00 | 10.00 | 10.00 | 10.00 | 10.00 |
| Microcrystalline cellulose | 10.00 | 10.00 | 10.00 | 10.00 | 10.00 |
| Vitamin premix[c] | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 |
| Mineral premix[d] | 2.00 | 2.00 | 2.00 | 2.00 | 2.00 |
| Phenylalanine[e] | 0.00 | 3.00 | 6.00 | 9.00 | 12.00 |
| Glycine[e] | 12.00 | 9.00 | 6.00 | 3.00 | 0.00 |
| Nutrient level[f] | | | | | |
| Moisture | 92.20 | 92.10 | 91.80 | 91.90 | 92.20 |
| Crude protein | 413.20 | 412.50 | 409.20 | 411.40 | 413.30 |
| Crude lipid | 182.80 | 178.60 | 176.50 | 180.40 | 183.30 |
| Crude ash | 37.80 | 37.30 | 37.70 | 37.00 | 37.50 |
| Gross energy (MJ/kg) | 21.73 | 21.45 | 21.48 | 21.62 | 21.50 |
| Phenylalanine (dry matter) | 18.20 | 20.30 | 22.90 | 26.40 | 30.01 |
| Phenylalanine (protein) | 44.39 | 49.51 | 55.85 | 64.38 | 73.41 |

[a]Dalong Feed Company, Harbin, China. [b]Compound amino acids composition: arginine 6.2 g, lysine 21.3 g, phenylalanine 9.0 g, histidine 4.6 g, valine 17.2 g, leucine 20.1 g, isoleucine 14.6 g, cysteine 1.5 g, methionine 6.6 g, proline 8.6 g, aspartic acid 17.8 g, tyrosine 8.2 g, alanine 14.2 g, glutamic acid 36.3 g, and glycine 7.2 g. [c]The vitamin premix provided the following per kg of diet: VC 100 mg, VE 60 mg, $VK_3$ 5 mg, VA 15,000 IU, $VD_3$ 3,000 IU, $VB_1$ 15 mg, $VB_2$ 30 mg, $VB_6$ 15 mg, $VB_{12}$ 0.5 mg, nicotinic acid 175 mg, folic acid 5 mg, inositol 1,000 mg, biotin 2.5 mg, and calcium pantothenate 50 mg. [d]The mineral premix provided the following per kg of diet: $MgSO_4 \cdot 7H_2O$ 2,000 mg, KCl 1 500 mg, $FeSO_4 \cdot 7H_2O$ 1,000 mg, $CuSO_4 \cdot 5H_2O$ 20 mg, $MnSO_4 \cdot 4H_2O$ 100 mg, $ZnSO_4 \cdot 7H_2O$ 150 mg, KI 3 mg, NaCl 500 mg, $CoCl_2$ 5 mg, and $Na_2SeO_3$ 3 mg. [e]Sigma Chemical Co., USA. [f]Nutrient levels were determined values.

a thiobarbituric acid reaction. The reaction was carried out at a colorimetric wavelength of 532 nm.

Homogenized intestinal samples were centrifuged at 6,000 × *g* for 20 min at 4°C in 10 volumes (w/v) of ice-cold saline. Subsequently, the supernatant was used for biochemical analysis using a lipase assay kit (LPS; A054-2-1) (30) and an amylase assay kit (AMS; C016-1-1) (31). Trypsin (A080-2-2) (32) content was determined by the UV colorimetric method; amylase (AMS) activity was determined by the starch iodine colorimetric method; lipase (LPS) content was determined by the colorimetric method, and protein content was determined by the Thomas Brilliant Blue method (33, 34). All kits were purchased from Nanjing Jiancheng Reagent Company and used according to the instructions.

## Histological examination

The mid-intestines of three fish in each replicate were randomly fixed in Bouin's solution for 48 h, then washed several times with water to remove the fixative, and embedded by conventional paraffin immersion. A microtome (KD 1508) was used to cut sections to a thickness of 6 μm. Sections were successively destained with ethanol, stained with hematoxylin and eosin, and finally sealed with neutral resin. After observation with a microscope (Leica MD 4000B), there were more than 10 intestinal slices in each group.

## Real-time quantitative PCR

Total RNA was isolated from intestinal tissues using RNAiso Plus (TaKaRa, China). The quality of the RNA was determined by analyzing the integrity of the RNA by agarose gel electrophoresis and confirming the absorbance ratio at A260/A280 nm between 1.8 and 2.0. The proposed RNA was reverse transcribed to cDNA using the PrimeScript™ RT reagent kit (TaKaRa, Dalian, China) and stored at −80°C in the refrigerator until use. Quantitative PCR (qPCR) was performed on a LightCycler® 480 thermal cycler (Roche, Germany) in a total volume of 10 ml using a Light Cycler® 480 SYBR Green I Master (Roche, Germany),

TABLE 2 Amino acids composition of the basal diets (air-dry basis, %).

| Amino acids | Groups | | | | |
|---|---|---|---|---|---|
| | G1 (1.82%) | G2 (2.03%) | G3 (2.29%) | G4 (2.64%) | G5 (3.01%) |
| Non-essential amino acid | | | | | |
| Asp | 1.90 | 2.51 | 3.03 | 2.77 | 2.79 |
| Ser | 1.40 | 1.08 | 1.36 | 1.25 | 1.71 |
| Glu | 5.27 | 5.26 | 5.20 | 4.73 | 5.45 |
| Gly | 2.40 | 2.98 | 2.34 | 1.78 | 2.04 |
| Ala | 2.53 | 2.60 | 3.15 | 2.98 | 2.84 |
| Cys | 0.76 | 0.89 | 0.85 | 0.91 | 0.69 |
| Tyr | 1.15 | 0.93 | 1.03 | 0.97 | 1.14 |
| Pro | 2.89 | 2.43 | 2.65 | 2.79 | 3.14 |
| Essential amino acid | | | | | |
| Thr | 1.19 | 1.00 | 1.28 | 1.16 | 1.59 |
| Val | 1.74 | 1.28 | 1.56 | 1.43 | 1.90 |
| Met | 1.31 | 1.76 | 2.05 | 2.23 | 2.18 |
| Ile | 1.23 | 0.84 | 1.04 | 0.95 | 1.27 |
| Leu | 4.20 | 3.68 | 3.35 | 3.03 | 3.27 |
| Phe | 1.82 | 2.03 | 2.29 | 2.64 | 3.01 |
| Lys | 2.26 | 1.64 | 2.10 | 1.93 | 1.60 |
| His | 0.82 | 1.18 | 0.97 | 0.89 | 1.22 |
| Arg | 1.95 | 1.40 | 1.91 | 1.76 | 1.93 |
| TAA | 34.82 | 33.49 | 36.16 | 34.20 | 34.76 |

Asp, aspartate; Thr, threonine; Ser, serine; Glu, glutamate; Gly, glycine; Ala, alanine; Cys, cysteine; Val, valine; Met, methionine; Ile, isoleucine; Leu, leucine; Tyr, tyrosine; Phe, phenylalanine; Lys, lysine; His, histidine; Arg, arginine; Pro, proline; EAA, essential amino acid; TAA, total amino acids.

following the manufacturer's protocol. All amplification reactions were compared using three replicates. All primer sequences in this experiment were referenced to the primer sequence of the *O. mykiss* gene published by Lee et al. (30) and Evenhuis et al. (35), as detailed in Table 3. β-Actin was used as an internal reference gene for the normalization of cDNA loading (36). The cycling conditions were 95°C for 30 s followed by 35 cycles of 95°C for 5 s, 59°C for 10 s, and 72°C for 30 s. Expression results were analyzed by the $2^{-\Delta\Delta CT}$ method.

## Calculation formula of growth performance

Weight gain rate (WGR; %) = $100 \times (W_t - W_0) / W_0$;
Condition factor (CF; %) = $100 \times W_t / Lt^3$;
Feed conversion ratio (FCR) = $W_f / (W_t - W_0)$;
Specific growth rate (SGR, %/d) = $100 \times (\ln W_t - \ln W_0) / t$;
Hepatosomatic index (HSI; %) = 100 × (liver weight (g) / body weight (g));
Viscerosomatic index (VSI; %) = 100 × (viscera (g) / body weight (g)).

$W_0$ is the initial body mass of the fish (g); $W_t$ is the terminal body mass (g); $L_t$ is the terminal body length of the fish (cm); $W_f$ is the feed intake (g); T is the test day (d) (37).

Statistical software SPSS 20.0 for Windows (SPSS Inc., Chicago, IL, USA) was used to conduct the one-way analysis of variance and Duncan's multiple comparisons of the data. All data were expressed as mean ± standard error (SE), with $p < 0.05$ used as the significant difference standard (36). The quadratic regression analysis of significant difference indices was carried out by Graphpad Prism 8.0 to determine the optimal demand range of phenylalanine for triploid *O. mykiss* under the condition of low fish meal (38). The bar charts in the article were also plotted using Graphpad Prism 8.0.

# Results

## Growth performance and somatic indices

As dietary phenylalanine levels increased, the WGR and SGR of triploid *O. mykiss* increased and then decreased, reaching a maximum in the 2.03% group and being significantly higher than the 3.01% group ($p < 0.05$) and the VSI ratio in the 2.03% group being significantly higher in all groups ($p < 0.05$) (Table 4). Primary, secondary, and tertiary linear regression equations were analyzed for WGR and SGR of triploid *O. mykiss* to determine the optimal addition of phenylalanine under low fishmeal feed conditions (Table 5). By comparing the $R^2$ values,

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TABLE 3 Primer sequences used for gene expression analyses.

| Genes | Primer sequences forward (5′-3′) | Primer sequences reverse (5′-3′) | Amplicon (bp) | Accession number | Primer efficiency (%) |
|---|---|---|---|---|---|
| β-Actin | F: GCCGGCCGCGACCTCACAGACTAC | R: CGGCCGTGGTGGTGAAGCTGTAAC | 73 | AC00648 | 99.65 |
| IL-1β | F: CTCTACCTGTCCTGCTCCAAA | R: ATGTCCGTGCTGATGAACC | 194 | AB010701.1 | 92.00 |
| IL-6 | F: CAATCAACCCTACTCCCCTCT | R: CCTCCACTACCTCAGCAACC | 91 | FR715329 | 96.00 |
| IL-2 | F: AGAATGTCAGCCAGCCTTGT | R: TCTCAGACTCATCCCCTCAGT | 69 | NM_001124657.1 | 95.00 |
| IL-10 | F: CGACTTTAAATCTCCCATCGAC | R: GCATTGGACGATCTCTTTCTTC | 70 | AB118099.1 | 96.00 |
| TNF-α | F: CCACACACTGGGCTCTTCTT | R: GTCCGAATAGCGGGAAATAA | 128 | AJ278085.1 | 96.00 |
| TGF-β | F: TCCGCTTCAAAATATCAGGG | R: TGATGGCATTTTCATGGCTA | 71 | AJ007836.1 | 95.00 |
| NF-κB | F: CAGGACCGCAACATACTGGA | R: GCTGCTTCCTCTGTTGTTCCA | 92 | XM_031794907.1 | 95.00 |
| Claudin-1 | F: TAGCATCCACGATCA | R: GAGCCTTCACTGGAGC | 124 | BK00876 | 96.00 |
| ZO-1 | F: CTGCTGGACGAAGGGA | R: GGCCTTTATCCTGCAT | 191 | HQ6560 | 95.00 |
| Occludin | F: ATGGCTCAATCTACAGG | R: GAGATACTGGTTGACCAACC | 102 | FR904483.1 | 95.00 |
| TOR | F: CCAAAGAGATGCAGAAGCCACA | R: CTCTCTCATACGCTCTCCCT | 178 | XM_020506200.2 | 98.00 |
| IGF-1 | F: ACTGTGCCCCTGCAAGTCT | R: CTGTGCTGTCCTACGCTCTG | 159 | M81904 | 93.00 |
| GH | F: CAAAGTGGGCATCAA | R: GTTCCTCCTGACGT | 139 | NM_0011246 | 96.00 |
| GHR | F: TCCCCTTCACCAGGA | R: TCATTCTGCAGTGGC | 148 | AB10083 | 97.00 |
| S6K1 | F: CCTCCTCATGACACCCTGCT | R: TCTTCTGGTCCGTTGGCAAA | 129 | XM_029674978.1 | 94.00 |
| 4EBP-1 | F: GGGGAACTCTGTTCAGCACA | R: AATGTTGGGGAGAGAGCACG | 117 | NM_004095 | 94.00 |

IL-1β, interleukin-1β; IL-6, interleukin-6; IL-2, interleukin-2; IL-10, interleukin-10; TNF-α, tumor necrosis factor-α; TGF-β, transforming growth factor-β; NF-κB, nuclear factor-κB; S6K1, S6 kinase 1; IGF-1, insulin growth factor 1; GH, growth hormone; GHR, growth hormone receptor; S6K1, 4EBP-1, eukaryotic initiation factor 4E-binding proteins-1.

TABLE 4 Effects of dietary phenylalanine levels on the growth performance and somatic indices of triploid *O. mykiss*.

| Items | Groups | | | | | |
|---|---|---|---|---|---|---|
| | G1 (1.82%) | G2 (2.03%) | G3 (2.29%) | G4 (2.64%) | G5 (3.01%) | SE |
| Initial body weight/g | 35.15 ± 1.72 | 39.09 ± 1.00 | 37.52 ± 3.92 | 36.87 ± 5.23 | 35.15 ± 3.78 | 0.04 |
| Final body weight/g | $89.33 \pm 2.58^{b}$ | $103.90 \pm 1.83^{d}$ | $95.37 \pm 3.04^{c}$ | $92.35 \pm 3.83^{bc}$ | $78.53 \pm 1.57^{a}$ | 1.23 |
| WGR/% | $154.14 \pm 3.35^{ab}$ | $165.80 \pm 4.37^{b}$ | $154.18 \pm 6.92^{ab}$ | $150.47 \pm 5.51^{ab}$ | $123.41 \pm 4.46^{a}$ | 1.53 |
| FCR | $1.35 \pm 0.18^{b}$ | $1.25 \pm 0.31^{a}$ | $1.50 \pm 0.15^{c}$ | $1.45 \pm 0.23^{d}$ | $2.19 \pm 0.55^{e}$ | 0.45 |
| SGR/(%/d) | $2.54 \pm 0.06^{ab}$ | $2.66 \pm 0.04^{b}$ | $2.54 \pm 0.07^{ab}$ | $2.50 \pm 0.06^{ab}$ | $2.23 \pm 0.04^{a}$ | 0.02 |
| SR (%) | 96.00 ± 4.00 | 96.00 ± 6.93 | 96.00 ± 6.93 | 93.33 ± 6.11 | 93.33 ± 8.33 | 1.02 |
| CF | 1.22 ± 0.09 | 1.23 ± 0.12 | 1.19 ± 0.03 | 1.16 ± 0.10 | 1.14 ± 0.09 | 0.03 |
| HIS (%) | $1.48 \pm 0.59^{a}$ | $2.12 \pm 0.51^{c}$ | $1.95 \pm 0.41^{bc}$ | $1.92 \pm 0.36^{bc}$ | $1.67 \pm 0.27^{ab}$ | 0.14 |
| VSI (%) | $11.49 \pm 1.94^{a}$ | $13.40 \pm 1.79^{b}$ | $12.76 \pm 1.89^{ab}$ | $13.39 \pm 1.44^{b}$ | $13.49 \pm 1.33^{b}$ | 0.24 |

Values are presented as mean ± SE ($n = 3$). Values in the same column with different superscript letters are significantly different ($p < 0.05$). WGR, weight gain rate; FCR, feed conversion rate; SGR, specific growth rate; PER, protein efficiency ratio; CF, condition factor; SR, survival rate; HSI, hepatosomatic index; VSI, viscerosomatic index.

TABLE 5 Linear modeling of the effect of dietary phenylalanine to low fish meal diets on weight gain rate and specific growth rate of triploid *O. mykiss*.

| | Primary folding line model | Quadratic linear model | Cubic linear model |
|---|---|---|---|
| WGR | $y = -39.398x + 246.47$ | $y = -47.636x^2 + 202.87x - 55.268$ | $y = 6.4319x^3 - 93.522x^2 + 309.85x - 136.76$ |
| $R^2$ | $R^2 = 0.9061$ | $R^2 = 0.9314$ | $R^2 = 0.9061$ |
| SGR | $y = -0.4074x + 3.498$ | $y = -0.3557x^2 + 1.3914x + 1.2493$ | $y = 0.1016x^3 - 1.2126x^2 + 3.7717x - 0.9027$ |
| $R^2$ | $R^2 = 0.914$ | $R^2 = 0.93$ | $R^2 = 0.931$ |

Equations and $R^2$ were calculated based on weight gain rates and specific growth rates, and data statistics were obtained from PRISM 8.

the quadratic equation provided good fits. From the regression analysis, it was shown that the WGR of triploid *O. mykiss* had a significant quadratic response to the increase in phenylalanine levels in the diet. The optimal phenylalanine requirement for triploid *O. mykiss* based on WGR was estimated to be 2.13% (Figures 1, 2).

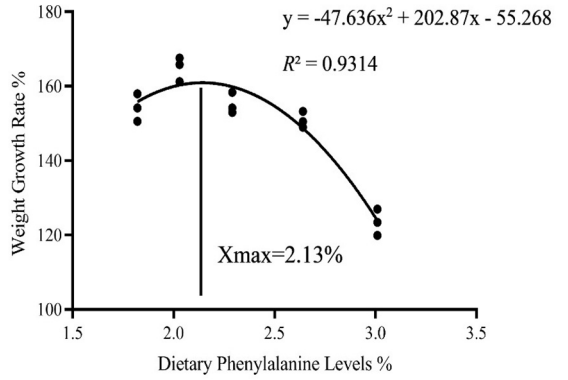


FIGURE 1
Quadratic regression analysis based on the weight gain rate of triploid *O. mykiss* fed experimental diets for 8 weeks.

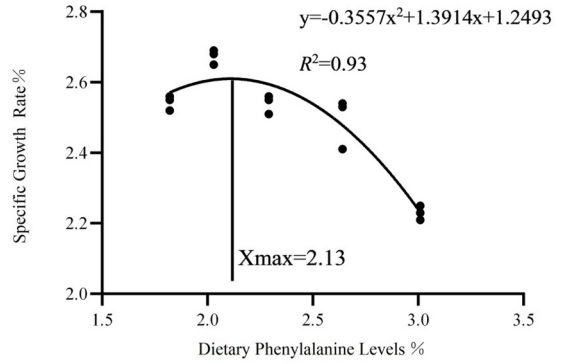


FIGURE 2
Quadratic regression analysis based on the specific growth rate of triploid *O. mykiss* fed experimental diets for 8 weeks.

## Effects of dietary phenylalanine levels on nutritional composition in triploid *O. mykiss* fed low fish meal diets

Whole-body crude protein levels peaked in the 2.03% group, which was significantly higher than the control group ($p < 0.05$). The highest whole fish lipid content was obtained when fed 2.03% phenylalanine and was significantly different from the other groups ($p < 0.05$) (Table 6). Meanwhile, dietary phenylalanine levels did not significantly affect the moisture and crude ash composition of triploid *O. mykiss* whole fish.

## Effects of dietary phenylalanine levels on amino acid composition in triploid *O. mykiss* fed low fish meal diets

Under low fish meal feed conditions, dietary phenylalanine levels significantly affected the amino acid profile ($p > 0.05$), except for valine ($p < 0.05$). Dietary phenylalanine had no significant effect ($p > 0.05$) on the levels of the first limiting amino acid, methionine, and the second limiting amino acid, lysine (Table 7). The tyrosine content at the end of the experiment was 1.78% (G1), 1.77% (G2), 1.71% (G3), 1.79% (G4), and 1.76% (G5), respectively. There was no significant difference between the treatment groups ($p > 0.05$).

## Effects of dietary phenylalanine levels on the antioxidant capacity in triploid *O. mykiss* fed low fish meal diets

The effects of dietary phenylalanine on antioxidant parameters in the serum and liver are displayed in Table 8. The serum SOD reached a maximum in the 2.03% group and was significantly higher than the control group ($p < 0.05$). There was no significant difference in liver SOD among different treatment groups ($p > 0.05$). Liver CAT peaked in the 2.03% treatment group and was significantly higher than in the other treatment groups ($p < 0.05$). There was no significant difference in serum MDA among treatment groups ($p > 0.05$), while liver MDA showed a trend of increasing and then stabilizing, reaching the maximum in the 2.29% group ($p < 0.05$). Serum and liver LZM reached the highest values at 2.03 and 2.29% of phenylalanine content, respectively, and were significantly different compared to the control group ($p < 0.05$).

## Effects of dietary phenylalanine levels on the intestinal digestive enzyme in triploid *O. mykiss* fed low fish meal diets

The effects of different dietary phenylalanine levels on the intestinal digestive enzyme activity of triploid *O. mykiss* are shown in Table 9. Trypsin activity was significantly higher in the 2.03% group than in the control group ($p < 0.05$). The AMS activity in the 2.03 and 2.29% groups was significantly higher than that in the control and other treatment groups ($p < 0.05$), but showed a gradual decrease with the increase in phenylalanine level. Dietary phenylalanine levels had no significant effect on the LPS activity of triploid *O. mykiss* ($p > 0.05$).

## Effects of dietary phenylalanine levels on the intestinal tissue morphology of triploid *O. mykiss*

Dietary phenylalanine levels had significant effects on the structural morphology of the intestine of *O. mykiss*. In Figure 3A (1.82% group), the intestinal villi were neatly arranged, and the surface striate margin was smooth. In Figure 3B (2.03% group), the length of the villi was longer and there were more cup-shaped cells and epithelial cells. In Figure 3C (2.29% group), the length of the villi reached its longest length and was significantly higher than the other treatment groups. However, the thickness of the muscle layer was thinner than that of the second group. In Figure 3D (2.64% group), the nucleus shift phenomenon began to appear, and the apical part of the villi started to shed. In Figure 3E (3.01% group), the intestinal muscular thickness was significantly lower, and the nuclei of the epithelial cells shifted significantly.

TABLE 6 Effects of dietary phenylalanine levels on the body composition of triploid *O. mykiss*.

| Indices | Groups | | | | | |
|---|---|---|---|---|---|---|
| | G1 (1.82%) | G2 (2.03%) | G3 (2.29%) | G4 (2.64%) | G5 (3.01%) | SE |
| Moisture | 70.01 ± 1.37 | 68.72 ± 1.78 | 68.78 ± 0.76 | 69.62 ± 0.65 | 69.30 ± 1.76 | 0.03 |
| Crude protein | 14.21 ± 1.50$^{a}$ | 15.28 ± 2.99$^{b}$ | 14.68 ± 2.97$^{ab}$ | 14.30 ± 1.56$^{a}$ | 13.17 ± 1.38$^{a}$ | 0.21 |
| Crude lipid | 10.46 ± 1.15$^{a}$ | 10.93 ± 1.14$^{b}$ | 10.71 ± 2.63$^{a}$ | 10.67 ± 0.79$^{a}$ | 10.52 ± 1.88$^{a}$ | 0.16 |
| Ash | 2.38 ± 0.02 | 2.50 ± 0.09 | 2.48 ± 0.13 | 2.49 ± 0.07 | 2.35 ± 0.04 | 0.04 |

Values are presented as mean ± SE ($n = 3$). Values in the same column with different superscript letters are significantly different ($p < 0.05$).

TABLE 7 Effects of dietary phenylalanine levels on the amino acid composition of whole triploid *O. mykiss*.

| Indices | Groups | | | | | |
|---|---|---|---|---|---|---|
| | G1 (1.82%) | G2 (2.03%) | G3 (2.29%) | G4 (2.64%) | G5 (3.01%) | SE |
| Essential amino acid | | | | | | |
| Thr | 2.68 ± 0.32 | 2.64 ± 0.11 | 2.28 ± 0.18 | 2.70 ± 0.08 | 2.68 ± 0.30 | 0.32 |
| Val | 2.79 ± 0.16$^{ab}$ | 2.76 ± 0.20$^{ab}$ | 2.39 ± 0.23$^{a}$ | 2.93 ± 0.19$^{b}$ | 2.59 ± 0.27$^{ab}$ | 0.03 |
| Met | 2.11 ± 0.01 | 2.10 ± 0.01 | 2.10 ± 0.01 | 2.11 ± 0.01 | 2.10 ± 0.01 | 0.04 |
| Ile | 2.47 ± 0.10 | 2.47 ± 0.16 | 2.29 ± 0.21 | 2.63 ± 0.14 | 2.39 ± 0.23 | 0.17 |
| Leu | 6.46 ± 0.25 | 6.36 ± 0.39 | 5.90 ± 0.71 | 6.56 ± 0.33 | 6.08 ± 0.68 | 0.36 |
| Phe | 2.41 ± 0.02$^{a}$ | 2.57 ± 0.19$^{a}$ | 2.79 ± 0.09$^{b}$ | 2.84 ± 0.12$^{b}$ | 3.03 ± 0.09$^{c}$ | 0.02 |
| Lys | 8.05 ± 0.34 | 8.13 ± 0.23 | 7.84 ± 0.72 | 8.26 ± 0.26 | 8.36 ± 0.01 | 0.13 |
| His | 1.38 ± 0.20 | 1.19 ± 0.16 | 1.21 ± 0.20 | 1.28 ± 0.15 | 1.11 ± 0.13 | 0.32 |
| Arg | 4.25 ± 0.35 | 3.96 ± 0.27 | 3.79 ± 0.58 | 4.17 ± 0.29 | 3.83 ± 0.40 | 0.07 |
| Non-essential amino acid | | | | | | |
| Asp | 9.03 ± 0.54 | 9.34 ± 0.23 | 9.29 ± 0.01 | 9.35 ± 0.22 | 9.33 ± 0.37 | 0.27 |
| Ser | 2.88 ± 0.29$^{b}$ | 2.64 ± 0.41$^{b}$ | 2.05 ± 0.30$^{a}$ | 2.65 ± 0.19$^{b}$ | 2.32 ± 0.26$^{ab}$ | 0.25 |
| Glu | 10.83 ± 0.14$^{b}$ | 10.72 ± 0.08$^{ab}$ | 10.59 ± 0.13$^{a}$ | 10.77 ± 0.09$^{ab}$ | 10.63 ± 0.08$^{ab}$ | 0.43 |
| Gly | 4.20 ± 0.65$^{b}$ | 3.58 ± 0.28$^{ab}$ | 3.24 ± 0.41$^{a}$ | 3.76 ± 0.38$^{ab}$ | 3.29 ± 0.37$^{a}$ | 0.06 |
| Ala | 4.24 ± 0.33 | 4.08 ± 0.01 | 4.15 ± 0.12 | 4.37 ± 0.25 | 4.10 ± 0.01 | 0.24 |
| Cys | 1.16 ± 0.03$^{b}$ | 0.88 ± 0.08$^{a}$ | 0.91 ± 0.09$^{a}$ | 1.10 ± 0.12$^{b}$ | 0.88 ± 0.08$^{a}$ | 0.04 |
| Tyr | 1.78 ± 0.04 | 1.77 ± 0.13 | 1.71 ± 0.23 | 1.79 ± 0.08 | 1.76 ± 0.12 | 0.13 |
| Pro | 2.66 ± 0.21$^{b}$ | 2.19 ± 0.29$^{ab}$ | 2.02 ± 0.38$^{a}$ | 2.26 ± 0.25$^{ab}$ | 1.90 ± 0.25$^{a}$ | 0.37 |
| Total | 69.57 ± 2.57 | 67.17 ± 2.68 | 64.19 ± 3.79 | 69.20 ± 3.10 | 65.32 ± 3.75 | 0.21 |

Values are presented as mean ± SE ($n = 3$). Values in the same column with different superscript letters are significantly different ($p < 0.05$).

The length of the villi and the thickness of the muscular layer are shown in Table 10. Villi length reached a maximum in the 2.29% treatment group and was significantly higher than that in the other treatment groups ($p < 0.05$). The thickness of the muscular layer was significantly higher in the 2.03% treatment group than that in the control group ($p < 0.05$).

## Expression of IGF-1, GH, GHR, TOR, S6K1, and 4EBP-1 in the intestine of *O. mykiss*

Dietary phenylalanine levels significantly affected the expression of intestinal growth-related genes in triploid *O. mykiss* ($p < 0.05$) (Figure 4). The expression levels of mTOR, downstream S6K1, and 4EBP-1 genes in the 2.03% treatment group reached their highest values, and there were significant differences with the 3.01% treatment group ($p < 0.05$). Similarly, GHR and GH gene expression levels were all highest in the 2.03% treatment group and significantly higher than the control group ($p < 0.05$).

## Expression of cytokines IL-1β, IL-2, IL-6, IL-10, TGF-β, TNF-α, and NF-κB in the intestine of *O. mykiss*

Dietary phenylalanine levels significantly affected the expression of interleukin (IL-1β, IL-2, IL-6, and IL-10) genes, TGF-β, and TNF-α in the intestine of triploid *O. mykiss* ($p < 0.05$) (Figure 5). IL-1β gene expression reached a minimum at a phenylalanine level of 2.03%, which was significantly lower than in the control group ($p < 0.05$). The expression of pro-inflammatory

TABLE 8 Effects of dietary phenylalanine levels on the antioxidant capacity of triploid *O. mykiss*.

| Indices | Groups | | | | | |
|---|---|---|---|---|---|---|
| | G1 (1.82%) | G2 (2.03%) | G3 (2.29%) | G4 (2.64%) | G5 (3.01%) | SE |
| SOD (U/mL) | | | | | | |
| Serum | $39.65 \pm 3.62^{b}$ | $46.48 \pm 6.24^{c}$ | $34.76 \pm 2.89^{b}$ | $36.83 \pm 3.16^{b}$ | $27.84 \pm 1.93^{a}$ | 1.25 |
| Liver | $10.22 \pm 1.58$ | $12.85 \pm 2.59$ | $10.94 \pm 2.66$ | $11.10 \pm 2.92$ | $12.76 \pm 1.09$ | 0.07 |
| CAT (U/mL) | | | | | | |
| Serum | $21.30 \pm 11.60^{a}$ | $37.51 \pm 18.22^{b}$ | $24.74 \pm 5.53^{a}$ | $32.30 \pm 7.70^{b}$ | $33.78 \pm 12.13^{b}$ | 1.56 |
| Liver | $17.33 \pm 1.23^{a}$ | $21.98 \pm 3.34^{b}$ | $17.59 \pm 2.98^{a}$ | $17.85 \pm 4.46^{a}$ | $21.35 \pm 2.33^{b}$ | 1.32 |
| MDA (nmol/mL) | | | | | | |
| Serum | $2.00 \pm 0.15$ | $1.76 \pm 0.23$ | $1.66 \pm 0.44$ | $2.00 \pm 0.51$ | $2.12 \pm 0.40$ | 0.11 |
| Liver | $5.15 \pm 0.88^{b}$ | $3.85 \pm 0.64^{a}$ | $3.56 \pm 0.33^{a}$ | $3.81 \pm 0.14^{a}$ | $3.94 \pm 0.22^{a}$ | 0.32 |
| LZM (U/mL) | | | | | | |
| Serum | $75.84 \pm 7.32^{a}$ | $94.67 \pm 11.00^{b}$ | $93.27 \pm 7.21^{b}$ | $73.12 \pm 8.01^{a}$ | $64.78 \pm 8.14^{a}$ | 0.44 |
| Liver | $20.05 \pm 8.40^{a}$ | $40.36 \pm 2.14^{b}$ | $54.67 \pm 8.84^{c}$ | $44.19 \pm 8.37^{bc}$ | $22.95 \pm 6.48^{a}$ | 1.89 |

Values are presented as mean ± SE ($n = 3$). Values in the same column with different superscript letters are significantly different ($p < 0.05$). SOD, superoxide dismutase; MDA, malondialdehyde; CAT, catalase; LAZ, lysozyme.

TABLE 9 Effects of dietary phenylalanine levels on the digestive enzyme of triploid *O. mykiss*.

| Indices | Groups | | | | | |
|---|---|---|---|---|---|---|
| | G1 (1.82%) | G2 (2.03%) | G3 (2.29%) | G4 (2.64%) | G5 (3.01%) | SE |
| Trypsin (U/mgprot) | $1{,}716.59 \pm 685.53^{a}$ | $4{,}602.81 \pm 717.13^{c}$ | $3{,}006.85 \pm 687.26^{b}$ | $2{,}874.80 \pm 650.82^{b}$ | $2{,}900.62 \pm 778.87^{b}$ | 78.76 |
| Lipase (U/gprot) | $43.39 \pm 13.27$ | $43.43 \pm 11.51$ | $46.07 \pm 6.79$ | $48.67 \pm 8.55$ | $44.55 \pm 6.32$ | 2.76 |
| Amylase (U/gprot) | $72.38 \pm 6.24^{a}$ | $106.82 \pm 12.06^{b}$ | $176.07 \pm 21.17^{c}$ | $85.03 \pm 3.93^{ab}$ | $92.56 \pm 12.41^{ab}$ | 8.97 |

Values are presented as mean ± SE ($n = 3$). Values in the same column with different superscript letters are significantly different ($p < 0.05$).

factors IL-2 and IL-6 was lowest in the 2.29 and 2.03% treatment groups, respectively, which was significantly different from the 3.01% treatment group ($p < 0.05$).

In triploid *O. mykiss* fed low fish meal diets, dietary phenylalanine levels had a significant effect on the expression of intestinal tumor necrosis factor (TNF-α) and nuclear factor-κB (NF-κB) genes ($p < 0.05$). TNF-β gene expression was lower in the 2.29% treatment group than in the control group ($p < 0.05$). TGF-β gene expression was highest in the 2.29% treatment group. TGF-β gene expression reached a maximum in the 2.29% treatment group. The nuclear transcription factor NF-κB also differed significantly among the groups. Compared to the other treatment groups, 2.03% of the treatment groups had significantly lower NF-κB mRNA expression ($p < 0.05$), while there was no significant difference among the G3–G5 groups.

### Effects of dietary phenylalanine levels on intestinal tight junction protein-related genes in triploid *O. mykiss* fed low fish meal diets

The expression of the intestinal tight junction protein gene was gradually increased as dietary phenylalanine levels ranged from 1.82 to 2.29%. The occludin gene in triploid *O. mykiss* showed a trend of increasing and then decreasing compared with the control group ($p < 0.05$) (Figure 6). The claudin-1 gene reached a maximum in the 2.03% groups and was significantly higher than that in the other groups ($p < 0.05$). The ZO-1 gene expression peaked in the 2.03% group, which was significantly different from the control group ($p < 0.05$).

## Discussion

### Effects of phenylalanine levels on the growth performance of triploid *O. mykiss*

Phenylalanine is an EAA for protein synthesis and growth stimulation in fish. Dietary phenylalanine can enhance fish feeding and increase the WGR and SGR of fish. This study showed that in low fish meal diets (phenylalanine level of 1.82%), the WGR and SGR of triploid *O. mykiss* showed a trend of increasing and then decreasing with increasing phenylalanine levels. Similar results were observed in Indian major carp and silver perch (12, 39, 40). In pomfrets (*Pampus punctatissimus*), it was found that the lack or excess of phenylalanine in the diet would lead to reduced growth performance and feed conversion rate (41). Phenylalanine deficiency and excess will disrupt the amino acid balance of the feed. The balance of amino acids in the feed will be disrupted, affecting the absorption and utilization of amino

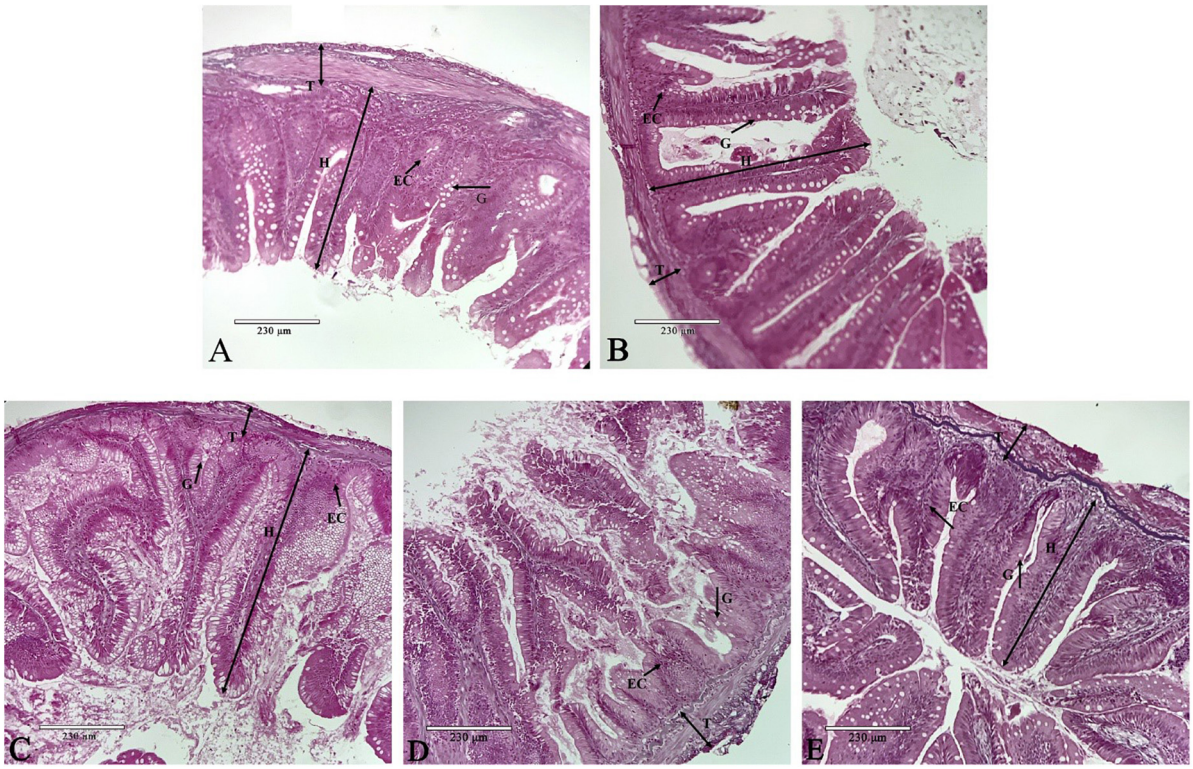


FIGURE 3
Intestine histology. Representative histological sections of the intestine from *O. mykiss* fed the different experimental diets. Scale bar, 230 μm. Panel **(A)** was G1 phenylalanine level in intestine tissue 100×; Panel **(B)** was G2 phenylalanine level in intestine tissue 100×; Panel **(C)** was G3 phenylalanine level in intestine tissue 100×; Panel **(D)** was G4 phenylalanine level in intestine tissue 100×; and panel **(E)** was G5 phenylalanine level in intestine tissue 100×.

**TABLE 10** Effects of dietary phenylalanine levels on the intestinal morphology of triploid *O. mykiss* (μm).

| Indices | Groups | | | | | |
|---|---|---|---|---|---|---|
| | G1 (1.82%) | G2 (2.03%) | G3 (2.29%) | G4 (2.64%) | G5 (3.01%) | SE |
| Villus length | $442.44 \pm 3.30^{a}$ | $511.88 \pm 18.31^{b}$ | $684.44 \pm 4.26^{c}$ | $464.97 \pm 36.59^{ab}$ | $465.11 \pm 15.93^{ab}$ | 8.86 |
| Muscular layer thickness | $88.15 \pm 5.38^{b}$ | $101.22 \pm 7.56^{b}$ | $61.53 \pm 4.17^{a}$ | $67.88 \pm 0.94^{a}$ | $67.55 \pm 8.38^{a}$ | 2.64 |

Values are presented as mean ± SE ($n = 3$). Values in the same column with different superscript letters are significantly different ($p < 0.05$).

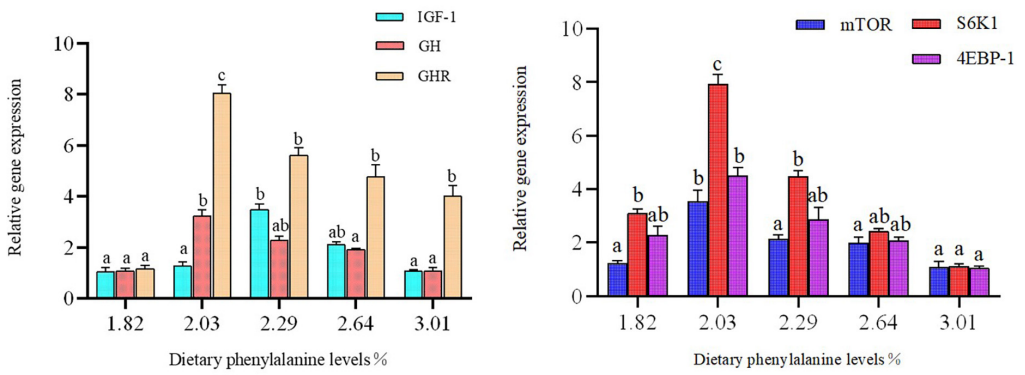


FIGURE 4
Growth gene expression of the intestine. Lowercase letters (a, b, or c) indicate a significant effect of liver growth on gene expression ($p < 0.05$). GH, growth hormone; IGF-1, insulin growth factor 1; GHR, growth hormone receptor; 4E-BP, eukaryotic initiation factor 4E-binding proteins; S6K1, S6 kinase 1.

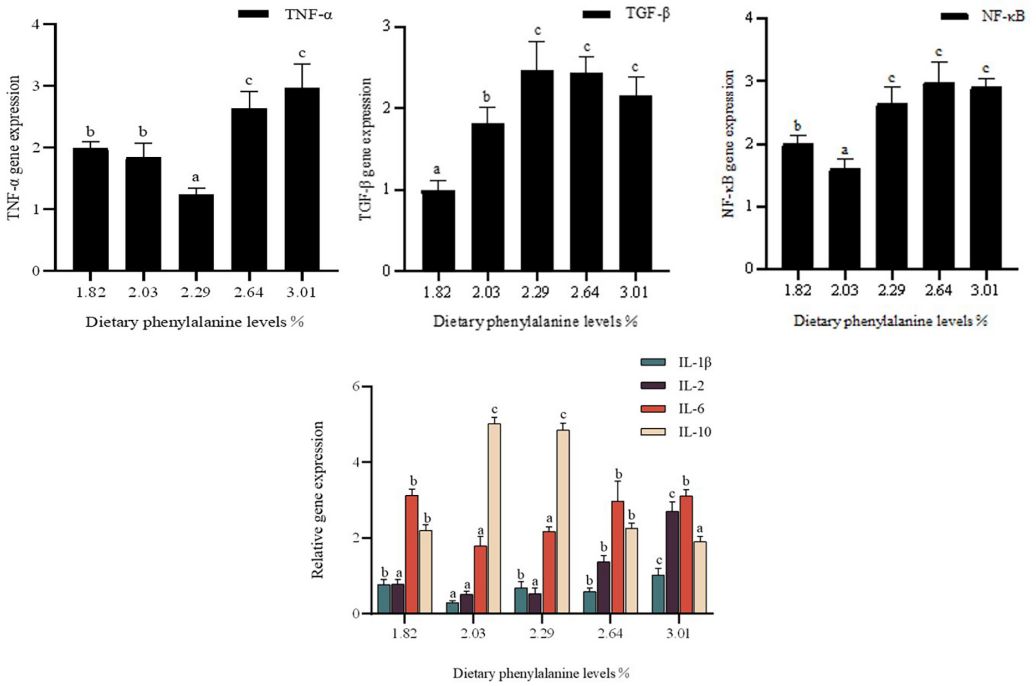


FIGURE 5
Immunity gene expression of the intestine. Lowercase letters (a, b, or c) indicate a significant effect of immune-related gene expression in the intestine ($p < 0.05$). IL-1β, interleukin-1β; IL-2, interleukin-2; IL-6, interleukin-6; IL-10, interleukin-10; TNF-α, tumor necrosis factor-α; TGF-β, transforming growth factor-β; NF-κB, nuclear transcription factors.

acids in the feed by fish, reducing the utilization of feed and protein synthesis, and thus inhibiting growth (42). It has been shown that the reduced growth performance of fish due to excess phenylalanine may be due to the energy consumption of excess phenylalanine in the body acting on deamidation, resulting in the oxidation of large amounts of phenyl pyruvic acid deposited in the body, producing toxic and even pathogenic effects (43). In Nile tilapia, it was shown that excess phenylalanine did not affect its growth performance (44). Other factors influenced by the cultural environment, such as water temperature, fish size, and amino acid composition, may also explain these disparities (45). It has also been suggested that the inhibitory effect of phenylalanine on fish growth may be because the body uses part of its energy to excrete nitrogen, which is because excess amino acids are easily degraded by the body and excreted in the form of nitrogen (46). However, the inhibitory effect of excess phenylalanine on fish growth is not conclusive, and more studies will be needed.

## Effects on the expression of genes related to growth in triploid *O. mykiss*

Intestinal health affects protein synthesis in the organism, which is regulated by TOR signaling molecules (47). When certain specific changes occur in the internal environment of the organism, the downstream effector protein S6K1 is regulated by TOR genes, thus participating in the regulation of cell growth, differentiation, and proliferation processes, while the downstream 4EBP-1 gene is also regulated by mTOR genes, regulating the growth process of the organism (48). When the S6K1 protein is activated in the cell, it phosphorylates several sites, including ribosomal protein S6, to promote the formation of the translation initiation complex (49). Silva found that IGF-1 is sensitive to changes in nutrients, especially amino acids (50). The relatively complex interaction between different hormones affects the growth regulation of hormones, among which GH, GHR, and IGF-1 are considered to be the most important growth-regulating genes. IGF-1 viability affects the secretion of growth hormones. The presence of growth hormone in the organism promotes the synthesis and release of this hormone, and the action of growth hormone on IGF-1 is mediated by the growth hormone receptor GHR, so GH-GHR binding is necessary to stimulate IGF-I synthesis and release (51). This study showed that the growth rate of triploid *O. mykiss* was slower when phenylalanine was deficient in the fish, but when phenylalanine was excessive, the WGR of *O. mykiss* had a more pronounced slowdown than when it was deficient. The expression of GH genes was highest at 2.29% phenylalanine level in the low fish meal diet, and the IGF-1 gene peaked at 2.03% and was significantly different from those of other groups. This has the same trend as the results obtained in Nile tilapia (52). The dietary amino acid imbalance was reported to reduce the expression level of the hepatic IGF-I gene in junco (*Rachycentron canadum*) (53) and Japanese seabass (*Lateolabrax japonicus*) (24). This is consistent with the findings of hybrid grouper larvae (*Epinephelus fuscointestinestatus*♀ × *Epinephelus lanceolatus*♂) (54), in which the treatment group with added complex protein had significantly higher daily feed intake and significantly higher rapamycin (TOR) liver target gene expression levels (55). In other amino acid studies, higher relative mRNA expression levels of rapamycin (TOR) and eukaryotic translation

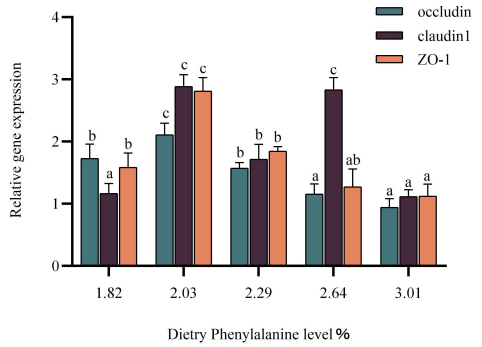


FIGURE 6
Tight junction protein gene expression of the intestine. Lowercase letters (a, b, or c) indicate significant effects of intestinal tight junction proteins relative to gene expression ($p < 0.05$).

initiation factor 4E-binding protein (4E-BP) were observed in 17.5 and 15.0 g/kg Leu diets (56), In the study of valine on rainbow trout growth gene expression, TOR mRNA and elF4E binding protein (4E-BP) expression were observed to be higher at 18.0 g/kg Val (57), The most significant effect of leucine on TOR and 4E-BP mRNA gene expression levels in rainbow trout was 13.5 g/kg (58), the same trend as the results of this experiment. In conclusion, dietary phenylalanine had an improved effect on the expression of growth-related genes in triploid *O. mykiss* with low fish meal diets. However, for our study, not explaining our results at the protein level is a shortcoming, and we will do more studies in the future to explain this mechanism and explain it in the discussion.

## Effects of dietary phenylalanine levels on the antioxidant capacity of triploid *O. mykiss*

Phenylalanine is a specific amino acid containing a phenyl ring structure that binds to hydroxyl radicals and eliminates hydroxyl radicals as well as reactive oxygen species (ROS) from the muscle. Oxidative stress occurs when the production of excess ROS overwhelms the antioxidant defense system, leading to cytopathology (59). The main enzymes that have the role of oxidant scavengers are SOD, catalase, and glutathione peroxidase. Non-enzymatic antioxidants include glutathione and other thiol compounds (60). In this experiment, dietary phenylalanine levels reduced the MDA content and increased the SOD content in the liver, thereby inhibiting oxidative damage caused by lipids and proteins. A previous study showed that phenylalanine could inhibit lipid peroxidation and protein oxidation by reducing ROS production in fish gills and that the phenylalanine deficiency group had significantly reduced resistance to superoxide anions and hydroxyl radicals. It was suggested that this could be related to the fact that the phenyl ring of phenylalanine can combine with hydroxyl radicals to form three hydroxylation products that can have a positive effect on scavenging free radicals (61). It has also been reported that the effect of phenylalanine on SOD activity may be related to its ability to promote the release of dopamine, which enhances extracellular SOD protein expression and cell surface SOD activity in rat astrocytes (62). However, whether dietary phenylalanine can stimulate the release of dopamine in fish has not been studied. Similar results were obtained in the present experiments in *Pagrus major* (63) but the serum CAT levels did not differ significantly in this experiment in triploid *O. mykiss*, which may be due to the different sensitivity of different fish to the stimulation of CAT in the intestine.

## Effects of dietary phenylalanine levels on the digestion of triploid *O. mykiss*

The ability of fish to digest and absorb nutrients is closely related to the activity of intestinal digestive enzymes. Phenylalanine improves the digestive capacity of fish by promoting the growth of the pancreas and intestine, which in turn improves the secretion of digestive enzymes and thus the digestive level of fish (64). In this experiment, the addition of phenylalanine to low fish meal diets significantly increased the intestinal trypsin and amylase activities of triploid *O. mykiss*, both of which reached a maximum in the 2.03% treatment group but did not have a significant effect on lipase activity. In Nile tilapia, there were significant differences in lipase activity but no significant differences in amylase activity in the intestine, which may be due to differences in the location of the digestive enzyme assay (65). But how phenylalanine affects the secretion of intestinal digestive enzymes has not been studied. In the gibel carp study, phenylalanine significantly increased hepatopancreas weight, intestinal length, intestinal weight, intestinal fold height, hepatopancreas, and intestinal trypsin, chymotrypsin, amylase, and lipase activities in gibel carp, and had significant effects on digestion-related indices in gibel carp (66). In contrast to the present experiment, grass carp (22), as an herbivorous fish, has a different ability to digest lipids than that of triploid *O. mykiss*.

## Effects of phenylalanine levels on immunity-related indices in triploid *O. mykiss*

Fish may convert phenylalanine into tyrosine, which can then be turned into melanin and catecholamines, which are significant immunomodulators with immunomodulatory activities (67). To date, IL-1β findings have been published in many fish species, including grass carp (*C. idella*) (68), *O. mykiss* (69), European sea bass (*Dicentrarchus labrax*) (70), Atlantic salmon (*Salmo salar*) (71), Nile tilapia (72) and channel catfish (*Ictalurus punctatus*) (73). IL-6 was discovered in Japanese flounder (*Paralichthys olivaceus*) (74), *O. mykiss* (75), gilthead seabream (*Sparus aurata*) (76), bluntnose seabream (*Megalobrama amblycephala*) (77) and roughy (*Larimichthys crocea*) (78). IL-8 has been cloned and identified in many fish species. These include Atlantic cod (*Gadus morhua*) (79), *O. mykiss* (80), Japanese flounder (81), and zebrafish (*Brachydanio rerio*) (82). This study showed that the dietary phenylalanine to low fish meal diets had a positive effect on regulating the expression of genes related to intestinal immunity in triploid *O. mykiss*. The pro-inflammatory factors IL-2 and IL-6 reached minimal values in the 2.03 and 2.29% treatment groups and were significantly higher than in the control group. IL-1β reached minimal values in the 2.03% treatment group and was significantly

lower than in the other treatment groups. The expression of IL-10, an anti-inflammatory factor, was highest in the 2.03 and 2.29% treatment groups and was significantly higher than in the other treatment groups. There are few reports on the effect of phenylalanine on intestinal inflammatory factors in fish. However, in humans, melanin can inhibit the production of cytokines such as IL-1β and IL-6 by human blood mononuclear cells because phenylalanine is a prerequisite for tyrosine, which can produce melanin, so we hypothesize that the gene expression of cytokines such as IL-1β, IL-6, and IL-2 in triploid *O. mykiss* is positively influenced by phenylalanine (83). Phenylalanine has been reported to reduce the number of peripheral blood lymphocytes in mice (*Mus musculus*). The production of peripheral blood lymphocytes in mice is stimulated by tetrahydrobiopterin, and phenylalanine promotes the production of tetrahydrobiopterin (84). Therefore, we speculate that phenylalanine also affects the expression of cytokines in triploid *O. mykiss* by affecting the number of its peripheral blood lymphocytes. However, the relevant studies on fish are few and need further validation.

### Effects of dietary phenylalanine levels on expression of tight junction protein-related genes in triploid *O. mykiss*

Fish intestinal health relies on a physical barrier composed of tightly linked proteins and epithelial cells. This study showed that either deficiency or excess of phenylalanine downregulated the expression of intestinal occludin, claudin-1, and ZO-1 in triploid *O. mykiss*. It has been shown that the function of the intestinal barrier is related to the inhibitory effect of phenylalanine on inflammatory factors. For instance, in human cells, IL-8 regulates the expression of occludin in vascular cells (85).

Tumor necrosis factor-α is also involved in tight junction protein expression regulation, which follows the same pattern as our experimental results. Lysine (86), arginine (87), methionine (88), and isoleucine (89) have all been studied for their effects on the expression of intestinal tight junction protein-related genes in fish, but less research has been done on phenylalanine. In grass carp, dietary phenylalanine could effectively improve the expression of intestinal tight junction proteins, with the highest expression of claudin-1, ZO-1, and occludin mRNA levels at 1.15% feeding (90). The expression of claudin-1, ZO-1, and occludin reached the highest values at a 2.03% phenylalanine level, which may be related to the different requirements of phenylalanine in the fish itself. As a result, adding appropriate phenylalanine to feed improves the regulation of tight junction protein expression in the organism and plays an important role in maintaining intestinal health.

## Conclusion

Dietary phenylalanine levels (2.03–2.64%) significantly increased the expression of intestinal growth-related genes and had a regulatory effect on the expression of immune-related genes in triploid *O. mykiss* fed a low fish meal diet (15%). Meanwhile, growth performance and body composition-related indicators have also been significantly improved. Using SGR and WGR as evaluation indices, the optimal requirement of phenylalanine for triploid *O. mykiss* was 2.13% by quadratic regression analysis. Based on the current research, the optimal phenylalanine addition level can be further explored to replace a fish meal with plant protein to provide a theoretical basis for the optimization of an artificial compound feed for triploid *O. mykiss*.

## Data availability statement

The original contributions presented in this study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

## Ethics statement

The animal study was reviewed and approved by the Committee for the Welfare and Ethics of the Laboratory Animals of Heilongjiang River Fisheries Research Institute, CAFS. Written informed consent was obtained from the owners for the participation of their animals in this study.

## Author contributions

SZ completed the experiments and wrote the manuscript. CW, HL, and YY provided the experimental design and financial support. YW, SLiu, and HJ had key roles in the data processing and mapping processes. SH and SLu contributed to the test shop equipment and water quality control. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The 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.

## Publisher's note



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# Nutritional profiling and contaminant levels of five underutilized fish species in Norway

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Exploring and making use of underutilized marine resources can be a sustainable approach to achieve future demands of fish consumption by the ever-growing population. Five species, namely European plaice (*Pleuronectes platessa*), European flounder (*Platichthys flesus*), lemon sole (*Microstomus kitt*), megrim (*Lepidorhombus whiffiagonis*), and thornback ray (*Raja clavate*), often captured as by-catch in Norway, were characterized for their nutritional value and potential accumulation of hazardous components. The proximate composition, protein profile, fatty acid profile as well as essential and toxic trace elements and polychlorinated biphenyls (PCBs) were analyzed. Digestible indispensable amino acid (DIAA) ratios and scores (DIAAS) and contributions of omega-3 fatty acids to the diet were calculated. Analysis on proximate composition revealed low fat contents of 0.74 to 1.25% and sufficient protein contents between 16.9 and 24% in the five species. Results of DIAA indicate a profitable distribution, with contributions exceeding the daily intake recommendations for an adult person related to a 200 g fillet. Moreover, findings on the distribution of eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) showed remarkable results, considering that the investigated species are lean fish. All five investigated fish exceed the recommended average daily intake level (AI) of EPA+DHA in a 200 g portion. As to toxic trace elements and PCBs, no significantly elevated levels were found considering a portion size of 200 g. Consequently, the nutritional quality of the investigated fish can be regarded as profitable with overall low potential health risks.

KEYWORDS

**nutritional profile, flatfish, underutilized fish, DIAAS, omega 3 fatty acids, risks and benefits, healthy**

## 1. Introduction

By 2050, the demand for food is projected to increase by 50% as the world's population is expected to reach 9.7 billion people (1, 2). At the same time, food production accounts for a quarter of total greenhouse gas emissions, and immediate action is needed to reduce climate gas emissions and counter climate change (3). Finding ways to produce more food, while at the same time reducing the climate impact of food, is a tremendous challenge in the years to come. Norway, being the country with the second-longest coastline globally shows a variety of marine species. Moreover, Norway is the second largest fish exporter (4). Nevertheless, only around 10%

of Norway's 220 marine species have been commercially utilized as food (5). A sustainable approach to achieve the future demand for fish can be to explore species that are considered as underutilized or classified as little utilized resources (LUR). A report on LUR species in Norway was published in 2011 and concluded that flatfish were among the species with the most significant potential for successful commercialization (6). Further research and investigations on flatfish were recommended to enable commercialization (6).

The excellent nutritional composition of fish and seafood in general has been reported in various studies and reviewed amongst others by Khalili Tilami et al. (7). Moreover, authorities have set recommendations to guarantee a satisfying intake of certain nutrients. FAO/WHO (8) and the European Food Safety Authority (EFSA) recommend a seafood consumption of 100 g and up to 300 g of fish per week, respectively, accounting for at least two meals a week to cover the recommended intake. High amounts of important long chain polyunsaturated fatty acids (LC-PUFAs) in fish are recognized for promoting overall human health due to their activities in physiological, molecular as well as cellular processes (9). Furthermore, marine proteins are recognized for their favorable nutritional value, due to high bioavailability and abundance of important peptides and essential amino acids. Studies focusing on the potential health benefits of marine proteins and hydrolysates are becoming increasingly prevalent due to their beneficial digestibility (7). Khalili Tilami et al. (7) mention that results indicate that fish proteins, peptides, and hydrolysates give improved health benefits somewhat comparable to marine lipids.

Next, to being a source of valuable macronutrients, fish contain essential trace elements like calcium and selenium. As a key indicator of bone density, calcium is crucial for the health of the skeleton and plays a vital role in many metabolic processes. Moreover, selenium deficiencies can lead to several diseases (7). Next to the importance of maintaining metabolic health in humans, selenium in fish is especially important because of its potential counter effects on methylmercury (10). Methylmercury is the methylated form of mercury, which naturally occurs in, e.g., volcanos and the atmosphere, but can also end up in environmental cycles if human caused sources like, e.g., fungicides, antiseptics or batteries are inappropriately discarded (11). Methylmercury is known to have several harmful impacts on human health. It is particularly problematic for pregnant women, as it can migrate across the placenta walls. High methylmercury exposure in pregnant women directly affects the neurodevelopment of the fetus (12). Moreover, bioaccumulation of persistent organic pollutants such as, e.g., polychlorinated biphenyls (PCBs) can pose serious health issues due to their persistence and toxicity to the human body (13).

The aim of the present study was to characterize four different flatfish (*Pleuronoectiformes*) species, and a ray (*Rajiformes*), whereof all are often captured as by-catch in Norway and regarded as underutilized species. More specifically, the study presents the chemical and nutritional profile of European plaice (*Pleuronectes* platessa), European flounder (*Platichthys flesus*), lemon sole (*Microstomus kitt*), megrim (*Lepidorhombus whiffiagonis*), and thornback ray (*Raja clavate*), including comparisons between species related to health promoting nutritional components. The analyses contained the proximate composition, total and free amino acids as well as fatty acid profile. Marine food sources in general are regarded as the main contributors to the intake of contaminants, which pose a potential risk for the consumers. Therefore, PCBs and trace elements, including both essential and toxic elements, were determined to support the safe consumption of these species.

# 2. Materials and methods

## 2.1. Raw material

The five fish species of interest were captured with purse seine between September 2020 and April 2021 by local fishermen at the Norwegian west coast. The catch occurred in area 2.a.2, according to the FAO Major Fishing Areas (14). The fish was gutted immediately after capture and kept on ice until the end of rigor mortis (41–57 h post-mortem). Fish were either filleted directly or frozen as whole (−80°C) and subsequently thawed before filleting. In order to have a thorough understanding of the chemical composition and identify any potential changes in the nutritional content within different body regions, muscle samples were taken from two fillets per flatfish. The two fillets, being the lower loin (LL) and upper belly (UB), of the flatfish samples were considered for analyses ($n_{\text{Flounder}} = 7 \times 2$, $n_{\text{Lemon sole}} = 5 \times 2$, $n_{\text{Plaice}} = 10 \times 2$, $n_{\text{Megrim}} = 5 \times 2$), as shown in Figure 1A. In contrast, only one fillet was kept for thornback ray ($n = 5$), as it consists of two central fillets (Figure 1B). All fillets were frozen directly and stored at −80°C until further use.

## 2.2. Proximate composition

Dry matter and ash content was determined following the AOAC 925.10 method (15). Samples were homogenized and between 1 and 2 g were weighed in duplicates in porcelain crucibles. The samples were placed in a dehydrator at 105°C for 24 h (TS8056; Termaks, Norway). After 24 h, the samples were placed in a desiccator to cool down to room temperature, weighed and water content was calculated according to equation 1. The dried samples were transferred to an ash oven and burned at 550°C for 20 h (B410; Nabertherm,Germany). The samples were placed in a desiccator to cool down, weighed and the inorganic matter was then determined following the principle of equation 1.

$$Water\ content\,(\%) = \frac{Sample_{wet} - Sample_{dried}}{Sample_{wet}} \times 100 \qquad (1)$$

Total crude protein content (%) was determined using the Kjeldahl method (15). A Kjeldahl apparatus (K-449 and K-375, Büchi Labortechnik, Schwitzerland) was used for measurements. The sample digestion and titration were carried out following the application manual No: 114/2013 of Büchi Switzerland. Briefly, sulfuric acid ($H_2SO_4$, 95–97%) and two Kjeldahl Tablets Eco (3.5 g $K_2SO_4$/0.105 g $CuSO_4 \times 5H_2O$/0.105 g $TiO_2$) were added to samples (1.5 g) before digestion. Digested samples were first neutralized with NaOH (32%, 15–90 ml) and $H_2SO_4$ (0.25 mol/l) was used as the titration solution subsequently. To determine the total protein concentration, a conversion factor of 6.25 × nitrogen (%) was applied (16).

Total lipids (%) were determined following the method of Bligh et al. (17). Samples (2 g ww) were weighed into chloroform-resistant tubes and chloroform was added. The solvent-sample mixture was

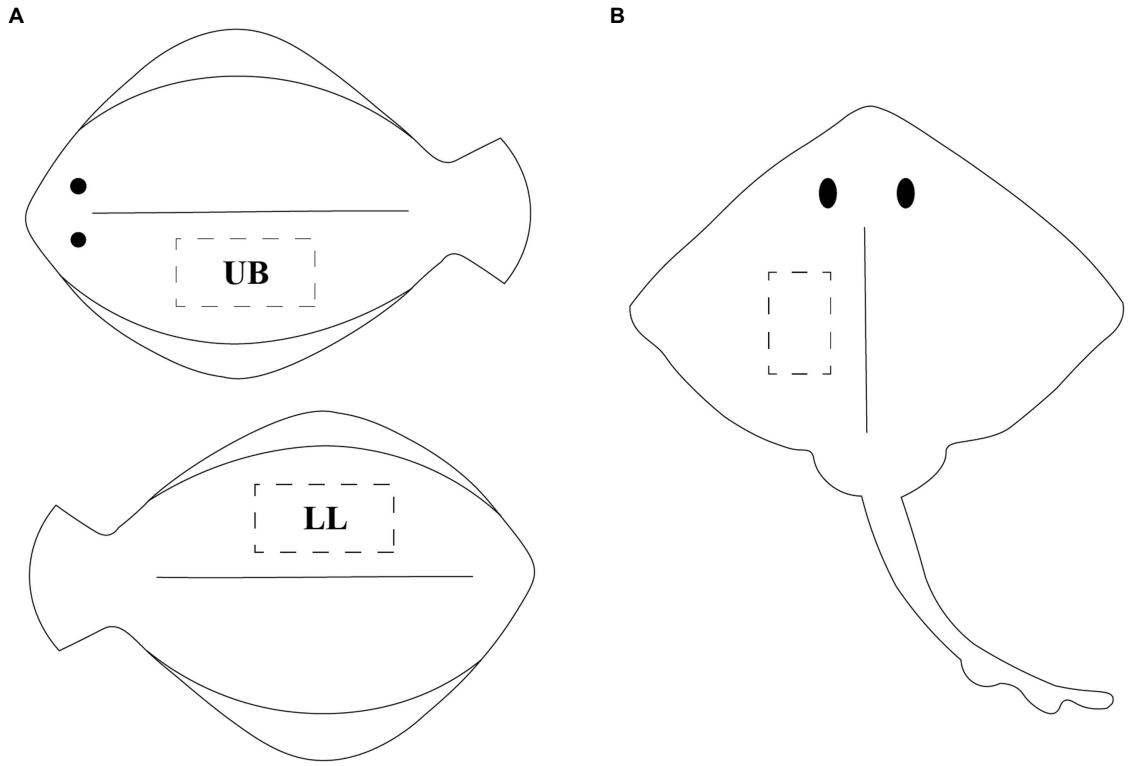


FIGURE 1
**(A)** Graphical illustration of flatfish, visualizing the two sampling points upper belly (UB) on the upper body part and lower loin (LL) on the back side of the fish; **(B)** illustration of ray, visualizing the sampling point of the central fillet.

subjected to extensive homogenization and centrifugation to achieve phase separation. The aqueous phase and chloroform-lipid phase were separated. Chloroform was evaporated from samples by applying liquid nitrogen. To accelerate the evaporation, the samples were placed on a heating block turned to 40°C (Stuart™ block heater type: SBH130D/3, Cole-Parmer, United States). The total lipids (%) were calculated according to Bligh et al. (17). The remaining chloroform phase containing lipids was frozen and stored at −80°C for fatty acid analysis.

## 2.3. Fatty acid composition

Fatty acids were prepared as methyl esters for analysis by gas chromatography. For fatty acid methyl ester (FAME) preparation, the method of Metcalfe et al. (18) was used. Chloroform phases containing lipids from individual fish were systematically merged to obtain five samples for thornback ray ($n = 5$), four samples for megrim ($n = 4$), and three samples each for lemon sole ($n = 3$) and flounder ($n = 3$). As these fish are very lean and individual fish were limited in size, it was necessary to merge samples to obtain at least 0.02 g of lipids per sample. Samples from European plaice were not merged due to bigger fish sizes, and 6 individuals ($n = 6$) were chosen for analysis. Nitrogen evaporation was conducted at 30°C until all chloroform was removed from the samples, and 3 ml KOH in methanol (0.5 M) was added to the samples and vortexed to saponify the lipids. Samples were incubated in a water bath at 70°C for 20 min, vortexed, and cooled on ice. Afterwards, 5 ml of boron trifluoride-methanol (14%, $BF_3$) was added to allow acid-catalyzed esterification of the fatty acids. The samples were re-incubated in the water bath at 70°C for 5 min and cooled on ice. *N*-butyl acetate (2 ml) was added, and the samples were shaken. Subsequently, saturated NaCl (around 1.5 ml), and two spatulas of powdered sodium sulfate ($Na_2SO_4$) were added to the samples, and the samples were rested at room temperature (21°C) to allow phase separation. Around 0.5 ml of hexane was added, and the lipid phase was then pipetted out and filtered using a 0.2 µm PTFE membrane (VWR International, United States) into GC vials.

The FAMEs were analyzed by gas chromatography (GC) using a GC apparatus (Agilent 6850, Agilent Technologies, United States). The samples (2–3 µl) were introduced by an evaporation injector (inlet: 260°C, pressure: 18.1 psi). Hydrogen was used as a carrier gas to pass the samples onto a polyethylene glycol column (HP-INNOWAX, i.D.: 0.25 mm; film: 0.25 µm, Merck Life Sciences, Norway), where FAMEs were separated at different times along the stationary phase. A flame ionization detector (FID) adjusted to 310°C was used to detect the samples. The oven program was set to a constant temperature of 160°C for 3 min, with an increase of 3°C/min to 240°C and held for 3 min.

Fatty acids were identified by comparing relative retention times (RRTs) of the external FAME standard mix containing 37 fatty acid methyl esters (Supelco 37 Component FAME Mix, Merck Life Sciences, Norway) with sample peaks. Chromatogram peaks, showing similar RRTs to the external standard were considered for determination. The intensity of each peak was calculated against the total intensity of FAMEs, to determine the percentage distribution of the individual fatty acids in each sample.

## 2.4. Protein profile

### 2.4.1. Amino acid distribution

Total amino acids were extracted from the samples following the method of Blackburn (19). Samples were freeze-dried for 22 h at −40°C and 13.3 Pa as a preparation for the analysis. Freeze-dried samples (80 mg) were weighed up in duplicates, and 1 ml of HCl (6 M) was added. The tubes were incubated for 22 h at 105°C to allow protein hydrolysis. Hydrolyzed samples were pH-neutralized by adding NaOH. The samples were filtered through a glass microfiber filter GF/C using suction, subsequently filled up to 10 ml with deionized water and suitably diluted. Diluted samples were filtered through 0.22 µm polyethersulfone filters (VWR International, United States) and transferred into HPLC vials.

Both free and total amino acids were analyzed by ultra-high-performance liquid chromatography (HPLC, UltiMate 300, Thermo Fisher Scientific, United States). As mobile phase, methanol and sodium acetate (0.08 M) with 2% tetrahydrofuran were applied. The HPLC was equipped with a Nova-Pak C18 column (WAT086344, particle size: 4 µm, 3.9 mm*150 mm, Waters Corp., United States), a TSP P400 pump and an injection valve (ultimate 3000WP injector). A pre-column derivatization step using the *o*-phtalaldehyde (OPA) method was applied and the flow rate was adjusted to 0.9 ml/min. After passing through the column, the amino acids were detected by fluorescence and recognized by a Dionex RF2000 detector. Alpha-aminobutyric acid (Aba) was used as an internal standard. Three amino acids were not analyzed: cysteine, proline, and tryptophan. The amino acids glycine and arginine were co-eluted in the analysis.

### 2.4.2. Free amino acid distribution

Free amino acids were extracted from the samples following the method of Osnes et al. (20). Approximately 2 g of frozen grated sample was placed into centrifuge tubes. Deionized water (10 ml) was added to the tubes, and the mixture was homogenized for 45 s to disrupt cells and release proteins (Ultra Turrax T25, Ika, Germany). The tubes were centrifuged for 3 min at 500 g at 4°C to obtain two phases (1700, Kubota, Japan). The soluble protein extract phase was taken out, and 1 ml of the extract was mixed with 0.25 ml of sulphosalicylic acid (10%, $C_7H_6O_6S$) to allow protein breakdown. The samples were shaken vigorously and placed in a fridge (4°C) for 30 min. After protein breakdown, the tubes were centrifuged for 10 min at 2700 g and 4°C (Megafuge 8R, Thermo Fisher Scientific, United States). The supernatant containing the free amino acids was suitably diluted. The diluted samples were filtered through 0.2 µm polyethersulfone membrane filters and 0.205 ml of the samples were transferred to vials before performing HPLC analysis as described in section 2.4.1.

## 2.5. Trace elements and polychlorinated biphenyls

For analyzing potentially elevated levels of contaminants in the samples, a variety of trace elements and polychlorinated biphenyls (PCBs) were chosen, and samples were pooled together. Each sample contained two individuals (three for European plaice) of same size, equally distributed and homogenized. Per species, two pooled samples ($n = 2$) were examined.

The samples were analyzed for 20 elements, including both toxic and essential trace elements such as Ag, Al, As, Ca, Cd, Co, Cr, Cu, Fe, Hg, K, Mg, Mn, Mo, Na, Ni, Pb, Se, V, and Zn. An inductive coupled plasma mass spectroscopy (ICP-MS, 8800 Triple Quadrupole; Agilent Technologies, United States) system was used. The system was linked to an autosampler (prepFAST M5, ESI, United States). To test the accuracy of the analysis, certified reference materials (CRM) were used, namely MODAS-5 (cod tissue, Nr. 0496) and MODAS-3 (herring tissue, Nr. 0958). The procedure of sample preparation, including microwave digestion and subsequent steps were previously described in detail by Kendler et al. (21) following the method of Sørmo et al. (22).

The analysis for PCBs included PCB-3, 8, 28, 52, 101, 118, 138, 153, 180, 195, 206, and 209, including the ICES-6 PCBs (PCB: 28, 52, 101, 138, 153, 180) and the dioxin-like PCB 118. A GC–MS system (7890A, Agilent Technologies, United States) was employed to detect PCBs. The system included split liner injection, an inert mass selective detector (5,975, Agilent Technologies, United States) and a Thermo TG 5MS column (length: 30 m; i.D.: 250 µm; film: 0.5 µm). A detailed description of the procedure can be found in Kendler et al. (21). The sample extraction followed the method described by Teunen et al. (23).

## 2.6. Nutritional quality parameters

### 2.6.1. Digestible indispensable amino acid score

The protein quality of a foodstuff can be determined by calculating the digestible indispensable amino acid score (DIAAS). For calculations on the quality of the distinct amino acid profiles of the five investigated fish species, the DIAAS as proposed by FAO (24) was considered and calculated.

The score is a product of the amino acid scoring pattern of the protein and the digestibility of these amino acids. The amino acid scoring pattern is related to how the amino acids in the protein correspond to the nutritional requirements set by FAO (24). A beneficial amino acid content is characterized by a high digestible indispensable amino acid (DIAA) content, which exceeds the nutritional requirements.

The DIAA reference ratios can be calculated for each DIAA from the amino acid content and the ileal digestibility, as seen in equation 2. IAA ratios above one are characterized by a high content of DIAA, which exceeds nutritional recommendations. DIAA ratios below one mean that the DIAA in the protein does not meet the recommendations. The lowest DIAA reference ratio is multiplied by 100 to obtain the DIAAS (24). Food with scores above 100 can be classified as "excellent" protein quality sources, scores between 75 and 100 can be classified as "good" protein quality sources, while scores below 75 can be regarded as "low" protein quality sources (24). Previous investigations on DIAAS in fish have determined them to be of excellent protein quality (25, 26).

$$DIAA\ reference\ ratio = \frac{mg\ of\ \ AA\ in\ 1\ g\ sample\ protein \times df}{mg\ of\ \ AA\ in\ 1\ g\ of\ \ reference\ protein} \quad (2)$$

Where:

df: true ileal digestibility factor for specific amino acids in fish as proposed by FAO (27). When specific digestibility factors were not

available for the given amino acid, the general digestibility factor for the protein was used.

reference protein: nutritional requirements set by FAO et al. (28).

### 2.6.2. Fatty acids

An estimation of the total amounts of fatty acids per 100 g edible fillet wet weight (ww) of the investigated species was conducted using equation 3. For the assessment of total fatty acids, published work from Weihrauch et al. (29) considering different lipid conversion factors in fish was applied, following the fatty acid conversion factor (FACF) as shown in equation 4.

$$g\ fatty\ acid\ per\ 100\ g\ fillets = weight\%\ FAME \times FACF \times TLC \quad (3)$$

Where:

Weight% FAME: results from FAME analysis, assuming the same as weight%-FA since marine lipids mainly consist of long-chain fatty acids (29).

FACF: fatty acid conversion factor (g FA/g lipid), from conversion factors proposed by Weihrauch et al. (29) calculated as in equation 4.

$$FACF = \frac{0.933 - 0.143}{TLC} \quad (4)$$

TLC: total lipid content as measured in g lipid per g fillet ww from the analysis on total lipids (17).

## 2.7. Statistical analysis

All statistical analyses were performed using Minitab 19[1] (Minitab Inc., United States). A Grubbs Outlier test with a significance level of $\alpha < 0.05$ was conducted to find outliers in the data set. Data were analyzed using univariate analysis of variance (ANOVA) combined with Tukey HSD *post hoc* test when significance was detected to investigate the differences between groups. Statistical differences were reported at the level of $\alpha < 0.05$. For flatfish representatives, analyses were carried out in 2 × 2 parallels (2 parallels for each UB and LL fillet; 4 in total per sample) and are presented as means ± standard deviation (SD) if not other stated. For thornback ray, the same analyses were performed in duplicates.

# 3. Results and discussion

In addition to species comparison, differences in the proximate and total and free amino acids composition among the UB and LL fillets were investigated for the four flatfish species. The UB fillets did not significantly differ from the LL for any of the species ($p > 0.05$). Hence, the data of the UB and LL fillets were combined, giving one mean value for each flatfish individual, which was further considered when presenting the results. This leads to the assumption that nutrients are equally distributed throughout the body regions of the investigated flatfish species. The findings correspond with our previous study on European plaice (21), where no significant difference in proximate and nutritional composition between muscle samples from upper and lower body fillets was found. Moreover, the results are in accordance with the study of Barbosa et al. (30) on megrim, which found no differences in lipid content between the upper and lower body fillets. The differences between fillets of upper and lower body were not investigated for thornback ray as its morphology differs from flatfish species, having only two main fillets.

## 3.1. Proximate composition

The proximate composition of flounder, lemon sole, megrim, plaice and thornback ray are shown in Table 1. Significant differences were observed for the species' ash, water, protein and lipid content. Megrim was found to have the lowest average water content of 79.2%, being significantly lower than flounder ($p = 0.003$). The measured water content for megrim equaled the values found by Afonso et al. (31), and Barbosa et al. (30), who showed values from 75–79%. Thornback ray showed a similar water content (80.1%) compared to lemon sole (81.4%) and plaice (80.5%), but differed from previous investigations by Colakoglu et al. (32) and Turan et al. (33) with water contents of 77%. The water contents of all investigated species are similar to those found by Karl et al. (34), investigating different flatfish species with average values ranging from 78.1 to 82.1%.

The ash content of the four flatfish representatives was between 1.0–1.25%, while fillets from thornback ray (0.9%) showed lower values of inorganic material. This was in line with previous investigations on flatfish, although previous studies on thornback ray found slightly higher ash values (1.1–1.4%) for this species (32, 33). Ash content in plaice is higher than previously investigated by Karl et al. (34) of 0.9% but similar to results from three different seasons by Kendler et al. (21) of values ranging from 1.07 to 1.28%. Plaice has a significantly higher ash content compared to flounder ($p = 0.005$) and thornback ray ($p < 0.001$).

For protein content, the differences were more significant between the investigated species. Megrim had a significantly higher protein content than lemon sole ($p = 0.036$), flounder ($p = 0.003$) and plaice ($p = 0.024$) with an average of 19.6%, being marginally higher than the studies by Barbosa et al. (30) with 16.6 to 18.6%. Lemon sole and flounder had a protein content of 16–18%, in line with previous investigations of plaice (16.6%) and yellowfin sole (16.0%) of Karl et al. (34). Significantly higher protein values were observed for thornback ray with 24.0%, being considerably higher than all investigated flatfish species ($p < 0.001$). The measured protein content of thornback ray was not in correspondence with previous studies and was 4–5% higher than observed in studies by Colakoglu et al. (32) and Turan et al. (33) with 18.6 and 20%, respectively. It must be mentioned that the proximate composition of thornback ray exceeds a total of 100%, which indicates an overestimation of the protein content measured by the Kjeldahl method using a conversion factor of 6.25. The Kjeldahl method measures the total nitrogen content, assuming approximately 16% nitrogen in proteins. However, other non-proteins in the cells also contain nitrogen, which can lead to an overestimation of the protein nitrogen in the food (35). Ray tissue contains around 350–400 mM urea, a nitrogen containing non-proteinaceous

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TABLE 1 Proximate composition of central fillets of flounder, lemon sole, megrim, plaice, and thornback ray.

| | Species | | | | | |
|---|---|---|---|---|---|---|
| Composition (%) | Flounder | Lemon sole | Megrim | Plaice | Thornback ray | *p*-value* |
| | $n=7$ | $n=5$ | $n=5$ | $n=10$ | $n=5$ | |
| Ash | $1.11\pm0.07^{b}$ | $1.04\pm0.05^{bc}$ | $1.10\pm0.04^{b}$ | $1.25\pm0.07^{a}$ | $0.94\pm0.10^{c}$ | <0.001 |
| Water | $82.1\pm1.2^{a}$ | $81.4\pm1.0^{ab}$ | $79.2\pm1.4^{b}$ | $80.5\pm1.4^{ab}$ | $80.1\pm0.8^{ab}$ | 0.004 |
| Proteins | $16.9\pm1.0^{c}$ | $17.5\pm0.7^{c}$ | $19.6\pm1.2^{b}$ | $17.6\pm1.1^{c}$ | $24.0\pm1.4^{a}$ | <0.001 |
| Lipids | $0.94\pm0.08^{ab}$ | $0.74\pm0.16^{b}$ | $0.98\pm0.16^{ab}$ | $1.25\pm0.47^{a}$ | $0.76\pm0.08^{b}$ | 0.015 |

Results presented as mean values ± SD. *ANOVA was applied to detect differences in proximate composition; where significant difference was detected ($\alpha<0.05$), a Tukey HSD *post hoc* test was applied. Values with different superscript (a, b) within a row are significantly different ($P<0.05$).

component, which might have been an interfering substance in the Kjeldahl analysis (36). Moreover, as the relative nitrogen content of amino acids fluctuates and the amino acid composition depends on the protein source, the assumption of 16% nitrogen content is rather general. Studies have shown that using a conversion factor of 6.25 can overestimate the total protein content of some foods (35, 37, 38). For this reason, attention has been given to creating conversion factors that are species/ food-specific (37, 38). Nevertheless, the established conversion factor of 6.25 is still widely used being officially recognized by the AOAC as a standard analytical method for protein determination, which makes results better comparable (39). The results suggest that protein contents of the four flatfish species are not overestimated, as the proximate composition (100%) is not notably exceeded and standard deviations are acceptable (1%).

Regarding the lipid content, all species can be considered as lean species, having values below 2%. Plaice was found to have significantly higher lipid values of in average 1.25% compared to lemon sole ($p=0.025$) with 0.74% and thornback ray ($p=0.036$) with 0.76% total fat content. The findings in this study show lower lipid contents for megrim (0.98%) compared to the study of Pastoriza et al. (40) finding a lipid content of up to 1.9%. Karl et al. (34) found lower lipid values for plaice of around 0.8%, but similar values of around 1% of other investigated flatfish. In a previous study of Kendler et al. (21), significant differences in the lipid content of plaice depending on fishing season were discovered ranging from 0.75 to 1.55%. For thornback ray, the measured lipid content of 0.76% was marginally higher than the finding of Turan et al. (33) of 0.5%, while much lower than the finding of Colakoglu et al. (32) of 3.4%. Two previous studies on deep-sea fish found that general deep-sea elasmobranchs like thornback ray had a lipid content of around 0.7 to 1.0%, which support the findings of the present study (41, 42).

## 3.2. Protein profile

Total amino acids (TAA) were investigated to determine the nutritional value of the proteins in the fish. The TAA results for the species are given in Table 2. The most abundant TAA for all species were leucine and lysine, as well as glutamic and aspartic acid, under physiological conditions in the form of glutamate and aspartate. The same abundant amino acids were found in an investigation of three flatfish species by Kim et al. (43), although showing higher amounts of glycine than in the present study. The contents of glutamate and aspartate can be regarded as overestimated as glutamine and asparagine were converted to these two amino acids during acid analysis (44). Consequently, asparagine and glutamine were detected in the lowest amounts in all investigated samples. Significant differences ($p<0.05$) were found for most of the amino acids between the five species. All species have a preferable distribution of indispensable amino acids (IAA), accounting for more than 50% of the TAA distribution. Megrim was found to have significantly higher amounts of IAA (7.24 g/ 100 g; $p=0.002$) compared to flounder (6.14 g/100 g), lemon sole (6.07 g/100 g), plaice (5.62 g/100 g), and thornback ray (5.88 g/100 g). Furthermore, megrim has comparably more total amino acids (13.80 g/100 g; $p=0.002$) than the other species. This was also found in three of the four species for the total protein content, with the exception of thornback ray, where an overestimated protein content is suspected. The amino acid determination by Blackburn (19) directly calculates the protein amount by considering only amino acid residues in the analysis and does not take into account possible interfering non-proteinaceous components. Amino acid hydrolysis can therefore be regarded as a good approach to determine the total amino acid content and gives an indication of the protein content of foods. However, the concentration of some of the amino acids might be lowered significantly due to the hydrolysis step prior to HPLC-analysis, which leads to an underestimation of the total protein content (35). Furthermore, it has to be mentioned that due to their instability during acid hydrolysis, cysteine and tryptophan were not determined in the applied method. Consequently, these two amino acids should be analyzed separately if the TAA measurement is used to indicate the total protein content.

The DIAA ratios were calculated and the DIAAS was calculated based on the DIAA ratios. The DIAA reference ratios are displayed in Table 3, and indicate whether all DIAA were present in the protein in adequate amounts to meet the requirements for adults set by FAO et al. (28). To calculate the ratios, the measured TAA levels were converted to mg/g protein for the different species as described in section 2.6.1. Scores above 1 indicate sufficient IAA levels, while ratios below 1 indicate insufficient levels. In addition, the DIAAS (%), was calculated, being the lowest amino acid ratio per species multiplied by 100 to convert the ratio to a percentage score. As indicated in Table 3, all amino acids show ratios above 1, despite methionine and cysteine (as combined values) in lemon sole (0.7), implying an excellent overall protein quality of the species. Cysteine and tryptophan were not analyzed in this study, which explains the low methionine + cysteine ratio, as it only consists of methionine. Ratios up to 2.5 for threonine were observed for thornback ray, megrim and lemon sole, pointing out the relevance of these species for a sufficient intake of indispensable amino acids. Moreover, DIAAS of over 100% were discovered for flounder (120%), megrim (120%), plaice (110%), and thornback ray

TABLE 2 Total amino acid contents (total-AA) in g/100g central fillets of flounder, lemon sole, megrim, plaice and thornback ray, showing indispensable (IAA) and non-indispensable amino acids (non-IAA).

| | Species | | | | | |
|---|---|---|---|---|---|---|
| Amino acids | Flounder | Lemon sole | Megrim | Plaice | Thornback ray | |
| | $n = 7$ | $n = 5$ | $n = 5$ | $n = 10$ | $n = 5$ | |
| **Indispensable*** | **Total-AA** | | | | | |
| | g 100 g$^{-1}$ | g 100 g$^{-1}$ | g 100 g$^{-1}$ | g 100 g$^{-1}$ | g 100 g$^{-1}$ | *p*-value*** |
| Histidine | 0.27 ± 0.04$^{b}$ | 0.31 ± 0.04$^{ab}$ | 0.36 ± 0.08$^{a}$ | 0.28 ± 0.06$^{ab}$ | 0.26 ± 0.007$^{b}$ | 0.026 |
| Isoleucine | 0.57 ± 0.06$^{b}$ | 0.59 ± 0.01$^{b}$ | 0.71 ± 0.05$^{a}$ | 0.53 ± 0.06$^{b}$ | 0.59 ± 0.01$^{b}$ | <0.001 |
| Leucine | 1.13 ± 0.12$^{b}$ | 1.06 ± 0.09$^{b}$ | 1.32 ± 0.09$^{a}$ | 1.03 ± 0.11$^{b}$ | 1.09 ± 0.02$^{b}$ | <0.001 |
| Lysine | 1.40 ± 0.08$^{b}$ | 1.37 ± 0.14$^{b}$ | 1.63 ± 0.09$^{a}$ | 1.24 ± 0.17$^{b}$ | 1.26 ± 0.03$^{b}$ | <0.001 |
| Methionine | 0.46 ± 0.05$^{a}$ | 0.20 ± 0.04$^{b}$ | 0.42 ± 0.12$^{a}$ | 0.37 ± 0.05$^{a}$ | 0.41 ± 0.04$^{a}$ | <0.001 |
| Phenylalanine | 0.56 ± 0.06$^{b}$ | 0.54 ± 0.05$^{b}$ | 0.68 ± 0.05$^{a}$ | 0.51 ± 0.05$^{b}$ | 0.57 ± 0.01$^{b}$ | <0.001 |
| Threonine | 0.69 ± 0.04$^{b}$ | 0.70 ± 0.1$^{b}$ | 0.85 ± 0.04$^{a}$ | 0.60 ± 0.06$^{c}$ | 0.69 ± 0.03$^{bc}$ | <0.001 |
| Valine | 0.63 ± 0.07$^{ab}$ | 0.58 ± 0.07$^{b}$ | 0.72 ± 0.09$^{a}$ | 0.58 ± 0.06$^{b}$ | 0.58 ± 0.01$^{b}$ | 0.008 |
| Σ IAA | 6.14 ± 0.69$^{b}$ | 6.07 ± 0.78$^{b}$ | 7.24 ± 0.42$^{a}$ | 5.62 ± 0.65$^{b}$ | 5.88 ± 0.11$^{b}$ | 0.002 |
| **Non-indispensable** | | | | | | |
| Asparagine | <0.01 ± <0.01 | <0.01 ± <0.01 | <0.01 ± <0.01 | <0.01 ± <0.01 | <0.01 ± <0.01 | 0.833 |
| Glutamine | 0.03 ± 0.007$^{b}$ | 0.01 ± 0.004$^{c}$ | 0.05 ± 0.008$^{a}$ | <0.01 ± <0.01$^{c}$ | 0.04 ± 0.01$^{b}$ | <0.001 |
| Arg/Gly** | 0.82 ± 0.04$^{ab}$ | 0.83 ± 0.1$^{ab}$ | 0.90 ± 0.08$^{a}$ | 0.73 ± 0.07$^{b}$ | 0.77 ± 0.02$^{ab}$ | 0.004 |
| Tyrosine | 0.52 ± 0.06$^{ab}$ | 0.49 ± 0.04$^{ab}$ | 0.56 ± 0.1$^{a}$ | 0.45 ± 0.05$^{b}$ | 0.41 ± 0.04$^{b}$ | 0.005 |
| Alanine | 0.89 ± 0.1$^{ab}$ | 0.74 ± 0.16$^{bc}$ | 1.0 ± 0.15$^{a}$ | 0.69 ± 0.06$^{c}$ | 0.79 ± 0.02$^{bc}$ | <0.001 |
| Aspartate | 1.39 ± 0.15$^{ab}$ | 1.35 ± 0.15$^{ab}$ | 1.61 ± 0.13$^{a}$ | 1.35 ± 0.17$^{b}$ | 1.32 ± 0.03$^{b}$ | 0.022 |
| Glutamate | 1.79 ± 0.19 | 1.83 ± 0.20 | 2.10 ± 0.15 | 1.95 ± 0.24 | 1.75 ± 0.05 | 0.049 |
| Serine | 0.74 ± 0.12$^{ab}$ | 0.85 ± 0.12$^{a}$ | 0.84 ± 0.14$^{a}$ | 0.60 ± 0.07$^{b}$ | 0.62 ± 0.01$^{b}$ | <0.001 |
| Σ Non-IAA | 5.64 ± 0.62$^{ab}$ | 5.61 ± 0.52$^{ab}$ | 6.56 ± 0.52$^{a}$ | 5.34 ± 0.57$^{b}$ | 5.29 ± 0.11$^{b}$ | 0.005 |
| Σ Total-AA | 11.78 ± 1.3$^{b}$ | 11.68 ± 1.3$^{b}$ | 13.80 ± 0.9$^{a}$ | 10.96 ± 1.2$^{b}$ | 11.17 ± 0.2$^{b}$ | 0.002 |

Results presented as mean values ± SD. *Tryptophan is not detected due to acid hydrolysis. **Arginine/Glycine could not be separated. ***ANOVA was applied to detect differences in total-AA, where significant difference was detected ($\alpha < 0.05$), a Tukey HSD *post hoc* test was applied. Values with different superscript within a row are significantly different ($P < 0.05$).

TABLE 3 Average total digestible indispensable amino acid ratios and scores (DIAAS) for central fillets of flounder, lemon sole, megrim, plaice, and thornback ray based on recommendations for adults set by FAO et al. (28).

| | | Digestible indispensable amino acid ratios | | | | |
|---|---|---|---|---|---|---|
| Amino acids | (28) Recommendations (mg/g protein) | Flounder | Lemon sole | Megrim | Plaice | Thornback ray |
| Histidine | 15 | 1.3 | 1.5 | 1.5 | 1.4 | 1.3 |
| Isoleucine | 30 | 1.5 | 1.5 | 1.6 | 1.5 | 1.6 |
| Leucine | 59 | 1.5 | 1.4 | 1.5 | 1.5 | 1.5 |
| Lysine | 45 | 2.4 | 2.4 | 2.4 | 2.3 | 2.3 |
| Methionine (+cys*) | 22 | 1.6 | 0.7 | 1.3 | 1.4 | 1.6 |
| Phenylalanine + tyrosine | 38 | 2.0 | 2.4 | 2.0 | 1.1 | 2.0 |
| Threonine | 23 | 2.3 | 2.5 | 2.5 | 2.2 | 2.5 |
| Tryptophan* | 6 | – | – | – | – | – |
| Valine | 39 | 1.2 | 1.1 | 1.2 | 1.2 | 1.2 |
| Total IAA | 277 | 1.7 | 1.7 | 1.7 | 1.7 | 1.7 |
| DIAAS (%)** | | 120% | 70% | 120% | 110% | 120% |

*Cysteine/Tryptophan not measured in analysis. Scores above 1.0 indicate contents higher than recommendations (green cells), scores below 1.0 indicate content lower than recommendations (red cells). DIAAS (%) ** calculated by multiplying the lowest DIAA ratio by 100.

(120%), stressing the high protein quality of the studied fish. Lemon sole shows a poorer DIAAS (70%), which is possibly due to the lack of data from cysteine.

In addition to their nutritional importance, amino acids are associated with taste when occurring unbound in the form of free amino acids (FAA) in biological systems. Figure 2 shows the FAA distribution (mg/100 g sample ww) of the five investigated species. FAA are grouped according to their distinctive flavor as described by Fuke et al. (45), Kirimura et al. (46) and Sarower et al. (47). FAAs have been identified as essential taste contributors in seafood (45–47). Glutamic acid, in the form of glutamate, glycine, and alanine are commonly identified among the most important taste contributors. Glycine and alanine are linked to sweetness, while FAAs such as valine, arginine, and methionine are linked to bitter taste in seafood (47). Aspartate and glutamate both provide a sour taste. However, especially relevant in seafoods, these amino acids also give an umami taste in the presence of sodium salts, such as the familiar monosodium glutamate (MSG). Phenylalanine and tyrosine also have a bitter taste, but can enhance the umami flavor (47). Related to the present study, significant differences between species were found for all FAAs, but arginine/glycine ($p = 0.114$; Arg/Gly as combined values), threonine ($p = 0.866$) and methionine ($p = 0.872$). Lysine showed significant differences amongst the species ($p < 0.001$), with flounder and megrim having notably higher values. Flounder, lemon sole and plaice were identified to have significantly higher levels of histidine ($p < 0.001$), while megrim and thornback ray show comparatively low values of 0.51 and 0.84 mg/100 g sample, respectively. The most prevalent FAA are allocated in the group of "Sweet" amino acids with arginine/glycine having the highest content, followed by lysine and alanine.

Referring to the TAA and FAA distribution of the five investigated species, high variances within the species were observed, which is expressed by relatively large standard deviations as shown in Table 2 and Figure 2. External factors such as sex, maturity, and feeding behavior can influence the chemical composition of fish. Moreover, seasonality can play an important factor, as pointed out in a previous study on chemical composition of European plaice caught during three seasons by Kendler et al. (21).

## 3.3. Fatty acid composition

The following sections (section 3.3 and 3.4) highlight the potential of the studied species as sources for eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in human nutrition. Flounder, plaice and thornback ray were found to have significantly higher total PUFA compositions than megrim with 50.7, 47.2, and 49.3%, respectively, as can be seen in Table 4. Significant differences were also found in the amount of present DHA. Thornback ray

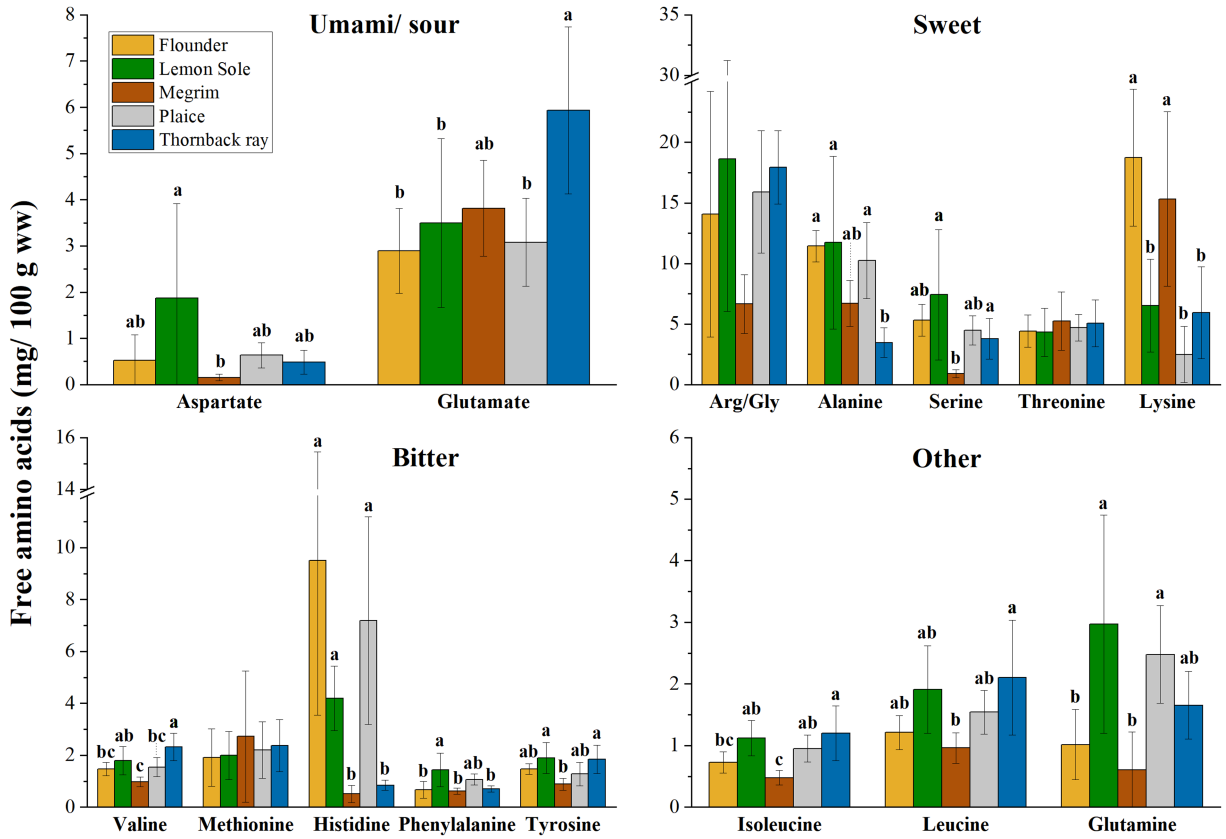


FIGURE 2
Free amino acids (FAA) in mg/100 g sample (ww), distribution grouped in taste perceptions umami/sour, sweet, bitter, and other. Error bars show SD. ANOVA was applied on species and each FAA; where significant difference between species was detected ($\alpha$<0.05), a Tukey HSD *post hoc* test was applied. Values with different letters (a, b) are significantly different ($p$<0.05). Asparagine is not displayed due to shallow contents (>0.1 mg/100 g).

TABLE 4 Fatty acid composition (% of total fatty acids w $w^{-1}$) of flounder, lemon sole, megrim, plaice, and thornback ray.

| | Species | | | | | |
|---|---|---|---|---|---|---|
| Fatty acids | Flounder | Lemon sole | Megrim | Plaice | Thornback ray | |
| | $n = 3$* | $n = 3$* | $n = 4$* | $n = 10$ | $n = 5$* | |
| **SFA** | % | % | % | % | % | *P*-value** |
| C14:0 | 1.58 ± 0.03$^{ab}$ | 1.45 ± 0.3$^{ab}$ | 2.68 ± 0.6$^{a}$ | 2.71 ± 1.4$^{a}$ | 0.29 ± 0.3 | 0.003 |
| C15:0 | 0.52 ± 0.3 | 0.37 ± 0.3 | 0.34 ± 0.2 | 0.17 ± 0.3 | 0.06 ± 0.1 | 0.110 |
| C16:0 | 20.9 ± 1.9$^{ab}$ | 23.86 ± 6.2$^{a}$ | 20.37 ± 3.0$^{ab}$ | 17.1 ± 2.6$^{b}$ | 26.0 ± 1.6$^{a}$ | <0.001 |
| C17:0 | 0.66 ± 0.2$^{ab}$ | 1.05 ± 0.1.0$^{a}$ | 0.26 ± 0.2$^{ab}$ | 0.17 ± 0.3$^{b}$ | 0.29 ± 0.39$^{ab}$ | 0.034 |
| C18:0 | 5.52 ± 0.7$^{ab}$ | 6.44 ± 1.1$^{a}$ | 4.64 ± 1.4$^{ab}$ | 3.50 ± 1.4$^{b}$ | 5.06 ± 0.3$^{ab}$ | 0.006 |
| Σ SFA | 29.19 ± 2.6$^{ab}$ | 33.17 ± 6.4$^{a}$ | 28.27 ± 3.3$^{ab}$ | 23.64 ± 2.8$^{b}$ | 31.66 ± 1.5$^{a}$ | <0.001 |
| **MUFA** | | | | | | |
| C14:1 | 0.04 ± 0.06$^{ab}$ | 0.07 ± 0.1$^{ab}$ | 0.22 ± 0.2$^{a}$ | 0.03 ± 0.09$^{b}$ | 0.00 ± 0.0$^{b}$ | 0.041 |
| C16:1 n7 | 2.88 ± 0.3$^{bc}$ | 1.81 ± 1.6$^{bc}$ | 4.78 ± 1.2$^{ab}$ | 6.93 ± 1.9$^{a}$ | 1.71 ± 0.09$^{c}$ | <0.001 |
| C17:1 | 0.13 ± 0.2 | 0.39 ± 0.4 | 0.31 ± 0.2 | 0.18 ± 0.3 | 0.00 ± 0.0 | 0.273 |
| C18:1 n7 | 2.37 ± 0.3 | 1.80 ± 1.6 | 2.66 ± 0.7 | 4.16 ± 2.4 | 3.55 ± 0.6 | 0.228 |
| C18:1 n9 | 10.08 ± 2.3 | 12.19 ± 4.9 | 13.68 ± 2.9 | 8.45 ± 3.4 | 8.36 ± 0.7 | 0.055 |
| C20:1 | 1.97 ± 0.3$^{ab}$ | 0.77 ± 0.9$^{b}$ | 5.26 ± 1.2$^{a}$ | 4.89 ± 2.7$^{a}$ | 1.27 ± 0.7$^{b}$ | 0.003 |
| C22:1 | 0.36 ± 0.6$^{ab}$ | 0.12 ± 0.2$^{ab}$ | 2.59 ± 2.0$^{ab}$ | 2.91 ± 1.9$^{a}$ | 0.00 ± 0.0$^{b}$ | 0.008 |
| Σ MUFA | 17.82 ± 2.3$^{b}$ | 17.16 ± 0.6$^{b}$ | 29.50 ± 5.8$^{a}$ | 27.53 ± 4.8$^{a}$ | 14.89 ± 1.8$^{b}$ | <0.001 |
| **PUFA** | | | | | | |
| C16:2 n4 | 0.67 ± 0.2 | 0.73 ± 0.6 | 0.56 ± 0.4 | 0.40 ± 0.5 | 0.12 ± 0.3 | 0.351 |
| C18:2 n6 (LA) | 2.95 ± 1.8$^{a}$ | 1.17 ± 1.0$^{ab}$ | 0.88 ± 0.6$^{b}$ | 0.40 ± 0.5$^{b}$ | 1.49 ± 0.2$^{ab}$ | 0.002 |
| C18:3 n3 | 0.77 ± 0.3 | 0.43 ± 0.4 | 0.72 ± 0.5 | 0.21 ± 0.3 | 0.10 ± 0.2 | 0.023 |
| C18:4 n3 | 0.39 ± 0.3 | 0.17 ± 0.3 | 0.51 ± 0.6 | 1.48 ± 1.9 | 0.00 ± 0.0 | 0.249 |
| C20:2 n6 | 0.11 ± 0.2$^{ab}$ | 0.39 ± 0.3$^{a}$ | 0.20 ± 0.1$^{ab}$ | 0.08 ± 0.1$^{ab}$ | 0.00 ± 0.0$^{b}$ | 0.037 |
| C20:4 n6 (AA) | 6.52 ± 1.6 | 8.94 ± 2.5 | 2.94 ± 1.8 | 4.11 ± 3.8 | 4.25 ± 0.6 | 0.07 |
| C20:4 n3 | 0.16 ± 0.3 | 0.16 ± 0.3 | 0.62 ± 0.4 | 5.9 ± 7.0 | 0.14 ± 0.3 | 0.115 |
| C20:5 n3 (EPA) | 13.24 ± 2.6 | 14.93 ± 3.0 | 6.73 ± 2.3 | 10.07 ± 8.9 | 3.96 ± 0.7 | 0.119 |
| C22:5 n3 (DPA) | 2.09 ± 0.4 | 2.49 ± 2.2 | 2.31 ± 0.3 | 4.68 ± 5.6 | 3.11 ± 0.7 | 0.744 |
| C22:6 n3 (DHA) | 23.84 ± 4.4$^{bc}$ | 14.72 ± 2.7$^{d}$ | 24.80 ± 2.2$^{b}$ | 19.83 ± 2.8$^{cd}$ | 36.17 ± 1.4$^{a}$ | <0.001 |
| Σ PUFA | 50.74 ± 3.3$^{a}$ | 44.12 ± 2.4$^{ab}$ | 40.26 ± 4.0$^{b}$ | 47.15 ± 4.0$^{a}$ | 49.34 ± 2.0$^{a}$ | 0.003 |
| Σ n3 | 40.49 ± 5.2$^{abc}$ | 32.9 ± 2.55$^{c}$ | 35.67 ± 3.9$^{bc}$ | 41.72 ± 4.4$^{ab}$ | 43.48 ± 1.8$^{a}$ | 0.005 |
| Σ n6 | 9.58 ± 2.2 | 10.50 ± 1.4 | 4.02 ± 1.1 | 4.92 ± 4.2 | 5.74 ± 0.6 | 0.022 |
| n3/n6 | 4.5 ± 1.8$^{bc}$ | 3.2 ± 0.6$^{c}$ | 9.3 ± 2.4$^{a}$ | 6.1 ± 2.3$^{abc}$ | 7.6 ± 0.9$^{ab}$ | 0.003 |
| Σ Others | 2.24 ± 3.9 | 5.55 ± 5.3 | 1.97 ± 2.1 | 1.67 ± 2.9 | 4.12 ± 1.4 | 0.303 |

Results presented as mean values ± SD. *Samples were merged in order to provide enough lipid phase to analyze for fatty acid composition. **ANOVA was applied to detect differences in fatty acid composition; where significant difference was detected ($\alpha < 0.05$), a Tukey HSD *post hoc* test was applied. Values with different superscript (a, b) within a row are significantly different ($P < 0.05$).

shows considerable higher values (36.2%) comparing to the other species and profoundly lower values were observed in lemon sole (14.7%).

Although the studied fish are categorized as lean species, the fatty acid composition is of high importance. Health promoting effects due to prevention of cardiovascular diseases (CVD), tumor cell proliferation and inflammation processes as well as beneficial effects on brain, retina and neurodevelopment in children are primarily attributed to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (9).

## 3.4. Nutritional value and essential elements

The total EPA and DHA intake when consuming the five investigated species was calculated to highlight how the species contribute to providing these indispensable FAs to human diet. The contribution to the daily recommendations of 250 mg EPA and DHA by the EFSA Panel on Dietetic Products et al. (48) are shown in Figure 3. Moreover, the average contribution of all IAA given as DIAAS by FAO (24) including the daily requirements of IAA for an

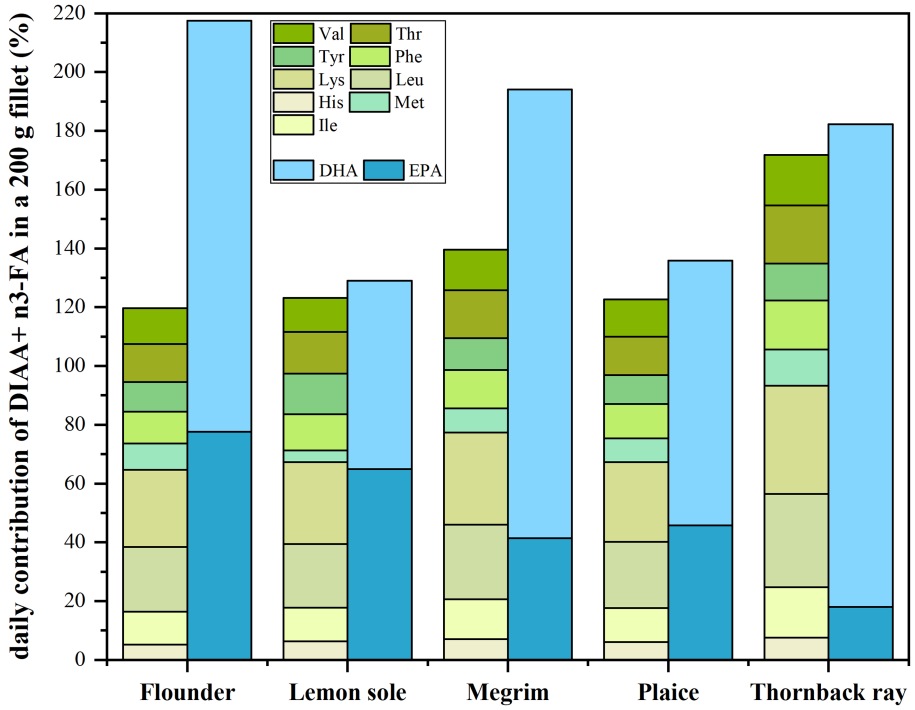


FIGURE 3
Given in green colors: average contribution of all DIAA to daily requirements for an 80 kg adult in a 200 g fillet portion by FAO, WHO (11); given in blue colors: average contribution of a 200 g fillet portion to cover the daily recommended intake of 250 mg DHA+EPA by EFSA (48).

80 g adult as proposed by FAO et al. (28) are shown in Figure 3. The values refer to a portion size of 200 g as recommended in a report on dinner serving sizes of foods by the Norwegian Food Authority (Mattilsynet) and were used for the calculations (49).

Figure 3 emphasizes on the nutritional value of the studied fish related to the content of DIAA as well as EPA and DHA in a 200 g fillet portion. The green bars show that the total required daily amount of DIAA for an 80 kg adult are met by all species. A fillet of 200 g of all species covers more than 120% of the total required DIAA, with thornback ray fulfilling the requirements to 172%. Nevertheless, to fully cover the daily intake of every individual indispensable amino acid (100%), a larger portion size than 200 g is needed for all investigated fish species. The daily recommended intake of, e.g., valine was restrictively covered in megrim (69%), hence a portion size of 371 g would be necessary to cover the daily demand of this particular amino acid (100%). Furthermore, valine is also the restrictive amino acid for thornback ray and flounder, where 54 and 61% of the daily demand is covered by a 200 g portion, respectively. In plaice, phenylalanine (53%) and in lemon sole, methionine (33%) are not fully covered with regards to a portion size of 200 g.

With regards to EPA and DHA, all investigated fish species contribute significantly to the daily suggested intake of 250 mg by EFSA (48). The relative contribution of DHA and EPA in 200 g fillets is shown in dark/light blue shading and was converted to mg/100 g edible portion using the conversion factors proposed by Weihrauch et al. (29) as described in section 2.6.2. Even tough being lean species, a 200 g portion of all of the five species contributes in average to more than 100% of the recommended average daily intake (AI) of EPA and DHA set by EFSA (48). The highest contribution was found in a 200 g portion of flounder (217%), followed by megrim (194%), thornback ray (182%), plaice (136%) and lemon sole (129%). With regards to the weekly recommended intake of 1.75 g EPA + DHA (250 mg × 7) this would mean a consumption of 3.2 portions of 200 g flounder, 3.6 of megrim, 3.8 of thornback ray, 5.2 of plaice and 5.4 portions of lemon sole, respectively. These results are highly relevant, as lean fish is usually not associated with providing sufficient levels of n3 fatty acids and the focus for covering n3 fatty acids was previously put on fatty fish such as salmon or trout in the past (50). All investigated species contain higher relative amounts of DHA, despite lemon sole, which has a 50:50 share of EPA and DHA.

Marine fish are good sources for both macro and trace elements, including minerals like calcium, magnesium or selenium, being vital for human health. All fish contain sufficient amounts of potassium (K) and magnesium as shown in Table 5. Significant differences between species were observed for the elements manganese ($p < 0.001$), magnesium ($p = 0.002$) and iron ($p = 0.047$). High values in selenium, ranging from 0.25 mg kg$^{-1}$ in thornback ray to 0.49 mg kg$^{-1}$ in lemon sole were found in this study. Compared to the study of Karl et al. (34) on different flatfish, selenium values ranging from 0.13 to 0.31 mg kg$^{-1}$ were reported. When setting dietary recommendations, the dietary reference value (DRV) is used. The DRV in this study refers to either the average requirement (AR), the population reference intake (PRI) or the adequate intake (AI), depending on the available data from the expert panel on Dietetic Products, Nutrition and Allergies from EFSA (51–60). Even though no significant difference ($p = 0.213$) in selenium content between species was detected, an effect on the contribution to meet the DRV is visible, given as % of DRV. Hence, a 200 g fillet of lemon sole covers the selenium intake to 140%, whereas a 200 g fillet of thornback ray reaches only 74.3% of the daily selenium coverage. Moreover, all five species are a good source of potassium, covering around 20% of the DRV. Differences in species are visible for the

TABLE 5 Essential elements and nutritional contribution of a 200g portion of fillet of flounder, lemon sole, megrim, plaice, and thornback ray.

| | | Species | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Flounder ($n$=2) | | | Lemon sole ($n$=2) | | | Megrim ($n$=2) | | | Plaice ($n$=6) | | | Thornback ray ($n$=2) | | | |
| Element | DRV (mg/day) | µg kg$^{-1}$ | EDI | % DRV | µg kg$^{-1}$ | EDI | % DRV | µg kg$^{-1}$ | EDI | % DRV | µg kg$^{-1}$ | EDI | % DRV | µg kg$^{-1}$ | EDI | % DRV | P-value* |
| | | | | | | | | | | | | | | | | | |
| Mn | 3.0 (51) | 70.5 ± 5.5$^{bc}$ | 0.014 | 0.47 | 94.8 ± 2.2$^{ab}$ | 0.019 | 0.63 | 107 ± 16.6$^{ab}$ | 0.021 | 0.71 | 39.8 ± 14.5□$^{c}$ | 0.008 | 0.27 | 145.8 ± 27.1$^{a}$ | 0.029 | 0.97 | <0.001 |
| Mo | 0.065 (52) | 0.7 ± 0.06 | 1×10$^{-4}$ | 0.22 | 1.2 ± 0.38 | 3×10$^{-4}$ | 0.38 | 0.06 ± 0.08 | 1×10$^{-5}$ | 0.012 | 0.87 ± 0.4□ | 2×10$^{-4}$ | 0.28 | 1.82 ± 1.5 | 4×10$^{-4}$ | 0.6 | 0.114 |
| Co | / | 2.5 ± 0.15 | 5×10$^{-4}$ | | 2.4 ± 1.12 | 5×10$^{-4}$ | | 0.52 ± 0.12 | 1×10$^{-4}$ | | 2.52 ± 1.1□ | 5×10$^{-4}$ | | 1.08 ± 0.23 | 2×10$^{-4}$ | | 0.098 |
| | | mg kg$^{-1}$ | | | mg kg$^{-1}$ | | | mg kg$^{-1}$ | | | mg kg$^{-1}$ | | | mg kg$^{-1}$ | | | |
| K | 3,500 (53) | 3,622 ± 315 | 724.4 | 20.7 | 3,411 ± 409 | 682.2 | 19.5 | 3,689 ± 4.3 | 737.8 | 21.1 | 3,493 ± 507 | 698.6 | 20.0 | 3,495 ± 154 | 699 | 20.0 | 0.847 |
| Na | 2000 (54) | 919 ± 71.4 | 183.8 | 9.2 | 730 ± 99.9 | 145.9 | 7.3 | 644 ± 201 | 128.9 | 6.4 | 1,085 ± 248 | 217 | 10.9 | 774.5 ± 86.0 | 154.9 | 7.7 | 0.191 |
| Mg | 300–350 (55) | 244 ± 28.2$^{ab}$ | 48.7 | 13.9 | 218 ± 34.6$^{bc}$ | 43.5 | 12.4 | 280 ± 6.7$^{a}$ | 56.0 | 16.0 | 188.7 ± 7.8$^{c}$ | 37.74 | 10.8 | 232.1 ± 2$^{abc}$ | 46.4 | 13.3 | 0.002 |
| Ca | 950–1,000 (56) | 151 ± 30.2 | 30.24 | 3.0 | 149 ± 35.7 | 29.8 | 3.0 | 146 ± 28.6 | 29.12 | 2.9 | 153.7 ± 59.1 | 30.74 | 3.1 | 172.5 ± 107 | 34.5 | 3.5 | 0.993 |
| Fe | 11–16 (57) | 0.8 ± 0.24 | 0.16 | 1.0 | 0.83 ± 0.25 | 0.17 | 1.0 | 0.74 ± 0.23 | 0.15 | 0.9 | 0.94 ± 0.25□ | 0.188 | 1.2 | 1.65 ± 0.5 | 0.33 | 2.1 | 0.047 |
| Zn | 12.7–16.3 (58) | 3.7 ± 0.2 | 0.74 | 4.5 | 3.18 ± 0.16 | 0.64 | 3.9 | 3.75 ± 0.08 | 0.75 | 4.6 | 3.90 ± 0.48□ | 0.78 | 4.8 | 3.23 ± 0.3 | 0.65 | 4.0 | 0.172 |
| Se | 0.070 (59) | 0.4 ± 0.02 | 0.07 | 100 | 0.49 ± 0.01 | 0.098 | 140 | 0.40 ± 0.02 | 0.08 | 114.3 | 0.40 ± 0.12□ | 0.08 | 114 | 0.26 ± 0.0 | 0.052 | 74.3 | 0.213 |
| Cu | 1.3–1.6 (60) | 0.2 ± 0.06$^{ab}$ | 0.034 | 2.1 | 0.16 ± 0.01$^{ab}$ | 0.032 | 2 | 0.2 ± 0.002$^{a}$ | 0.038 | 2.4 | 0.11 ± 0.02□$^{b}$ | 0.022 | 1.4 | 0.2 ± 0.03$^{ab}$ | 0.032 | 2 | 0.031 |

$n$ = pooled samples constituting of multiple individuals per sample. Results presented as mean values ± SD; DRV, dietary reference values; EDI, estimated daily intake, mg/200 g fillet. □Results extracted from a previous study by Kendler et al. (21). *ANOVA was applied to detect differences in trace elements, where significant difference was detected ($\alpha$ < 0.05), a Tukey HSD post hoc test was applied. Values with different superscript ($^{a,b}$) within a row are significantly different ($p$ < 0.05). DRV is expressed as PRI (population reference intake) or AI (adequate intake), dependent on the relative scientific opinion from EFSA as given in the references (51–60). For EDI calculation, average DRV values of Mg, Fe, Zn, and Cu were used.



10.3389/fnut.2023.1118094

TABLE 6 Toxic trace elements of flounder, lemon sole, megrim, plaice, and thornback ray.

| | Season | | | | | |
|---|---|---|---|---|---|---|
| Toxic trace elements | Flounder | Lemon sole | Megrim | Plaice□ | Thornback ray | |
| | $n = 2$ | $n = 2$ | $n = 2$ | $n = 6$ | $n = 2$ | $P$-value* |
| | µg kg$^{-1}$ | µg kg$^{-1}$ | µg kg$^{-1}$ | µg kg$^{-1}$ | µg kg$^{-1}$ | |
| V | 5.54 ± 4.41 | 10.20 ± 10.57 | 2.54 ± 1.69 | 12.4 ± 16.3 | 0.67 ± 0.05 | 0.756 |
| Cr | 14.71 ± 2.99$^{bc}$ | 37.89 ± 6.28$^{ab}$ | 12.56 ± 2.63$^{bc}$ | 7.6 ± 2.1$^{c}$ | 56.36 ± 29.12$^{a}$ | 0.002 |
| Ni | 16.52 ± 2.4$^{a}$ | 9.29 ± 0.06$^{b}$ | 8.92 ± 0.8$^{b}$ | 2.01 ± 0.6$^{c}$ | 10.75 ± 1.07$^{b}$ | <0.001 |
| Ag | 0.24 ± 0.1 | 0.35 ± 0.49 | 0.13 ± 0.03 | 0.19 ± 0.1 | 0.51 ± 0.02 | 0.316 |
| Cd | 0.19 ± 0.01$^{b}$ | 0.13 ± 0.03$^{b}$ | 0.12 ± 0.05$^{b}$ | 0.17 ± 0.09$^{b}$ | 0.58 ± 0.05$^{a}$ | <0.001 |
| Pb | 2.42 ± 0.67$^{ab}$ | 2.98 ± 0.17$^{a}$ | 3.50 ± 0.37$^{a}$ | 0.81 ± 0.5$^{c}$ | 1.20 ± 0.2$^{bc}$ | <0.001 |
| Hg | 39.78 ± 1.74 | 58.05 ± 2.29 | 70.77 ± 10.69 | 112.9 ± 54.1 | 174.37 ± 169 | 0.350 |
| | mg kg$^{-1}$ | mg kg$^{-1}$ | mg kg$^{-1}$ | mg kg$^{-1}$ | mg kg$^{-1}$ | |
| As | 19.87 ± 7.05 | 105.3 ± 65.6 | 3.51 ± 0.63 | 58.9 ± 28.3 | 29.94 ± 9.33 | 0.051 |
| Σ toxic elements | 19.95 ± 7.1 | 105.4 ± 65.6 | 3.60 ± 0.6 | 59.05 ± 28.4 | 30.18 ± 9.5 | |

$n$ = pooled samples constituting of multiple individuals per sample. Results presented as mean values ± SD. □Results extracted from a previous study by Kendler et al. (21). *ANOVA was applied to detect differences in trace elements and PCBs; where significant difference was detected ($\alpha < 0.05$), a Tukey HSD *post hoc* test was applied. Values with different superscript ($^{a, b}$) within a row are significantly different ($P < 0.05$).

magnesium contribution, where a 200 g portion of plaice covers 10% of the DRV and megrim contributes up to 16% of the daily recommended intake.

## 3.5. Contaminants

Being demersal fish species, *Pleuronoectiformes* and *Rajiformes* are more likely to accumulate PCBs and hazardous trace elements than other fish species (13). Therefore, when frequently eating flatfish or ray, the matter of food safety must be considered. However, both intrinsic and environmental variables have a role in the bioaccumulation of hazardous as well as beneficial compounds (31). Individuals different in sizes and sexes, exhibit different concentrations in trace elements and contaminants, due to a variety in habitat and migration behaviors. In this study several toxic trace elements were determined (Table 6). The analysis on PCBs, including both non-dioxin and dioxin-like PCB congeners, show values lower than the detection limit (LOD) for flounder, lemon sole, megrim, and thornback ray. Hence no significant accumulation of any of the investigated PCB congeners were detected in those four species. Concerning plaice, traces of PCB 3, 52, 101, 118, 138, 153, and 180 were detected, as previously reported in the study of Kendler et al. (21) that looked into seasonal differences. A reason for the lower PCB values compared to plaice could be the lower fat content as well as overall lower fish size in the three other flatfish and the thornback ray. With regards to toxic trace elements, significantly different values between the species were detected for the elements chromium ($p = 0.002$), nickel ($p < 0.001$), cadmium ($p < 0.001$) and lead ($p < 0.001$). The highest accumulation of chromium and cadmium were found in thornback ray with 56.36 ± 29.12 µg kg$^{-1}$ and 0.58 ± 0.05 µg kg$^{-1}$ respectively. Large varieties within species can be seen for all elements, with particular great SD in the mercury content of plaice (112.9 ± 54.1 µg kg$^{-1}$) and thornback ray (174.37 ± 169 µg kg$^{-1}$). Despite differences between species as well as individuals, the maximum levels of cadmium (0.1 mg kg$^{-1}$), lead (0.3 mg kg$^{-1}$) and mercury (0.5 mg kg$^{-1}$) set by the EC (61) are not exceeded. When considering arsenic as potential hazardous component, organic and inorganic arsenic must be differentiated. Inorganic arsenic is the toxic form and according to Sloth et al. (62) a maximum of 1% of total arsenic in marine species is found in the form of hazardous arsenite and arsenate. The calculations in the previous study of Kendler et al. (21) on arsenic content in European plaice were followed for the other four species in this study. Considering the suggestion of 1% inorganic arsenic (58), it is safe to consume the recommended portion size by the Norwegian Food Authority (49) of 200 g of each of the five investigated species.

# 4. Conclusion

This study highlighted the nutritional composition of flounder, lemon sole, megrim, plaice, and thornback ray. The distribution and contribution of DIAA and the two main n3 fatty acids EPA and DHA show remarkable nutritional quality in all five species. A 200 g fillet portion of each of the five species covers the total DIAA and the recommended average daily intake of n3 fatty acids for an adult person. The nutritional score, emphasizing on DIAA and n3-fatty acids, can be regarded as profitable with good overall quality of all five fish. This study emphasized on the benefits of consuming these five species, mainly in the form of n3-fatty acids, DIAA, and essential minerals, but also investigated potential hazardous components. Potential risk factors in the form of PCBs and toxic trace elements were analyzed and have shown only minor bioaccumulation of single elements below the suggested upper intake limits. In conclusion, our study provides important insights into the nutritional profile of five underutilized fish species in Norway. However, it is important to note that there were some limitations in our study, including an incomplete TAA profile analysis, by not covering tryptophan and cysteine, as well as a potential overestimation of the protein content due to the chosen conversion factor. To further improve the understanding of the amino acid composition and total protein content, future studies should include cysteine analysis and focus on evaluating species-specific

conversion factors Future work should also put a stronger focus on assessing the risks and benefits of these fish that come with increased consumption. This is necessary to promote a safe consumption and integrate these fish, which have not yet been considered commercially in Norway, into the diet.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Author contributions

SK: conceptualization, methodology, formal analysis, investigation, and writing – original draft. FT: investigation and writing – original draft. AJ: conceptualization, methodology, and supervision. JL: conceptualization, methodology, supervision, project administration, and funding acquisition. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The 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.

## Publisher's note



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# n-3 PUFA poor seafood consumption is associated with higher risk of gout, whereas n-3 PUFA rich seafood is not: NHANES 2007–2016

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**Background and aims:** Gout, the most prevalent inflammatory arthritis, has undesirable effects on the quality of life. Omega-3 polyunsaturated fatty acids (n-3 PUFA) has a strong link with anti-inflammatory impacts. However, whether the harmful effects of seafood in relation to gout may vary owing to different levels of n-3 PUFA in seafood is still unclear. It was the goal of this study to examine the relationship between n-3 PUFA poor/rich seafood consumption and gout.

**Methods:** Between 2007 and 2016, five NHANES cycles were performed, with 12,505 subjects having complete data for gout and two 24-h dietary intake interviews. The 24-h dietary recalls were utilized to evaluate dietary habits. Gout was defined based on questionnaires. Weighted logistic regression models were conducted to investigate the association between n-3 PUFA poor/rich seafood consumption and gout. Moreover, subgroup analysis was utilized to estimate the stability of results. Covariates including age, gender, race/ethnicity, income, education, body mass index, chronic kidney disease, diabetes mellitus, hypertension, smoking status, and drinking status were stratified in different models.

**Results:** In the fully adjusted model, each unit of increase of n-3 PUFA poor seafood intake was associated with an 8.7% increased risk of gout (OR = 1.087, 95% CI: 1.039, 1.138, $P < 0.001$), whereas, no correlation was found between n-3 PUFA rich seafood consumption and gout. It also provided a proof-of-concept regarding the potential for n-3 PUFA rich seafood to counteract harmful effects of purines in relation to gout. A dose-response analysis showed that there was a non-linear relationship between n-3 PUFA rich seafood intake and the risk of gout in the female group.

**Conclusion:** Findings suggest that n-3 PUFA poor seafood consumption is associated with higher risk of gout, whereas n-3 PUFA rich seafood is not.

KEYWORDS

**n-3 PUFA poor/rich seafood consumption, gout, inflammation, National Health and Nutrition Examination Survey, nutrition**

# Introduction

Gout is an inflammatory crystal arthritis, the most important pathological characteristic of which is monosodium urate deposition in the joints. The incidence of gout has been stably increasing to 0.58–2.89 per 1,000 person-years worldwide with the development of the world economy and lifestyle changes (1). Additionally, gout episodes have undesirable effects on the quality of life (2).

Recent studies have revealed a strong link between omega-3 polyunsaturated fatty acids (n-3 PUFA) and anti-inflammatory impacts (3–5). Numerous systematic reviews and meta-analysis have noted that n-3 PUFAs exhibited anti-inflammatory effects on multiple non-communicable diseases, including systemic inflammatory response syndrome (3), colorectal cancer (4), type 2 diabetic mellitus (5), bipolar disorder (6), mental disorders (7), polycystic ovary syndrome (8), heart failure (9), hypertension (10), and rheumatoid arthritis (11). With respect to gout, experiments *in vitro* noted that n-3 PUFAs could inhibit the NLRP3 inflammasome in monocytes *via* downstream effects of a two-signal initiation system (12, 13), including suppression of nuclear factor kappa-B (NF-κB) *via* Toll-like receptor 4 and Toll-like receptor 2 (14) and assembly of the inflammasome and activation of caspase-1 (15). Clinically, a case control study showed that low serum n-3 PUFA levels were connected with frequent gout flares (16). Although it is well established that most seafood, which typically contains large quantities of purines, leads to increased risk of gout (17, 18), whether the harmful effects of seafood in relation to gout may vary owing to different levels of n-3 PUFA in seafood is still unclear. According to the US Department of Agriculture (USDA) Dietary Research Nutrition Database, the seafood component is divided into: seafood that are high in n-3 PUFA and seafood that are low in n-3 PUFA. Whether n-3 PUFA poor seafood or n-3 PUFA rich seafood can affect gout remained to be elucidated.

Hence, the preliminary aim of this study was to determine the relationship between n-3 PUFA poor/rich seafood consumption and gout in US adults using data from the National Health and Nutrition Examination Survey (NHANES). Our study was the first large-scale cross-sectional study evaluating n-3 PUFA poor/rich seafood consumption and gout, which could shed new light on gout management.

# Materials and methods

## Study population

Potential subjects in the present study were selected from 2007 to 2016 cycle of NHANES. NHANES is a periodic, nationally representative health study conducted by the Nation Center for Health Statistics (NCHS) at the Centers for Disease Control and Prevention (CDC) aimed at evaluating individuals' health and nutritional status. NHANES was approved by the NCHS Ethics Review Board. All subjects provided written informed consent. All data in this study are publicly accessible at http://www.cdc.gov/nchs/nhanes/.

Participants aged <20 years old ($n$ = 21,387), without complete information about dietary data ($n$ = 6,489), missing data on gout ($n$ = 1,131), or missing baseline condition ($n$ = 9,076) were excluded, leaving a total of 12,505 subjects for the present analysis (Figure 1).

## Measurements and covariation assessment

### Dietary assessment

A total of 24-h dietary recalls were utilized to calculate individual dietary intakes, which have been examined by the Nutrition Methodology Working Group (19). To ensure precise food recall and alleviate the respondent burden, eligibility criteria included subjects who had both two valid 24-h dietary recalls.

Definition of seafood was founded on the USDA Dietary Research Nutrition Database. Seafood high in n-3 PUFA included anchovy, herring, mackerel, salmon, sardine, shark, trout, and bluefin and albacore tuna. Seafood low in n-3 PUFA included catfish, clams, cod, crabs, crayfish, croaker, eel, flounder, haddock, lobster, mussels, octopus, oyster, perch, pollock, scallop, shrimp, snapper, tilapia, tuna (other than bluefin and albacore), and turtle.

### Gout

Similar to previous NHANES reports (20), all participants were asked "Has a doctor or other health professional ever told you that you had gout?" and then classified as non-gout participants and gout participants.

## Covariables

### Sociodemographic characteristics

According the current articles, the following variables were collected during the household interview, including age,

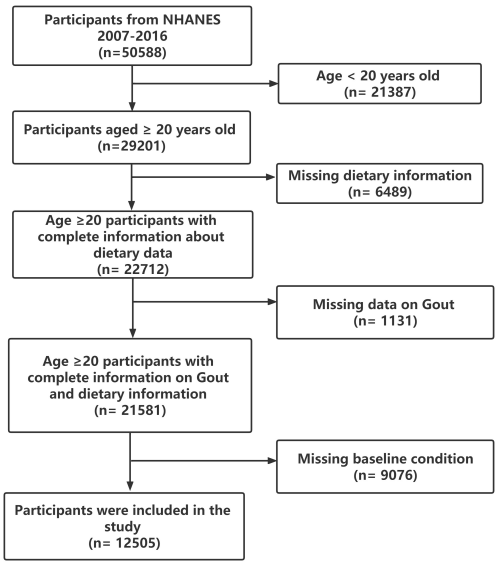


FIGURE 1
Study flowchart.

gender, race/ethnicity, education level, and family poverty income ratio (PIR). Race/ethnicity was categorized into Mexican American, non-Hispanic Black, non-Hispanic White, other Hispanic, and other race (including multi-racial) based on NHANES classification (21). Education level was classified as less than high school, high school graduate/General Education Development (GED); some college/Associate of Arts (AA) degree and college graduate or more (22). PIR was utilized to evaluate family income. PIR was categorized into three strata (<1, 1–3, and ≥3) and defined as poor, near poor, and not poor, respectively (23).

### Body mass index status

Body mass index (BMI) was calculated as the weight divided by the square of height (kg/m$^2$). BMI <18.5 was considered as underweight, 18.5–24.9 as normal, 25–30 as overweight, and ≥30 as obese (24).

### Smoking status

Smoking behavior was classified as never smoker, former smoker, and current smoker based on their answers to questionnaire about smoking more than 100 cigarettes in their life and whether they had quit smoking (25).

### Drinking status

Drinking behavior was categorized as mild drinking (≤1 drink per day for women or ≤2 drinks per day for men on average over the past 12 months), moderate drinking (1–3 drinks per day for women or 2–4 drinks per day for men on average over the past 12 months), and heavy drinking (≥4 drinks per day for women or ≥5 drinks per day for men on average over the past 12 months) (26).

### Chronic kidney disease

We used the Chronic Kidney Disease Epidemiology Collaboration formulate to assess estimated glomerular filtration rate (eGFR). Chronic kidney disease (CKD) was defined as eGFR <60 ml/min/1.73 m$^2$ or urine albumin creatinine ratio (UACR) >30 mg/g (27).

### Hypertension

Hypertension was defined as systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg based on the average of three measurements of all subjects' blood pressure (28).

### Diabetes mellitus

Diabetes mellitus (DM) was defined as self-reported diabetes, the use of diabetes medication or insulin, glycohemoglobin (HbA1c) >6.5%, fasting glucose ≥7.0 mmol/L, random blood glucose ≥11.1 mmol/L, or 2-h oral glucose tolerance test (OGTT) blood glucose (mmol/L) ≥11.1 (29). Impaired fasting glucose (IFG) was defined as fasting plasma glucose concentration ≥5.6 and ≤6.9 mmol/L (30). Impaired glucose tolerance (IGT) was defined as a plasma glucose level ≥7.8 and ≤11.0 mmol/L at 2 h after OGTT (30).

## Statistical analysis

All statistical analyses were performed following CDC guidelines for analysis of NHANES data. A suitable sample weight was utilized to calculate all statistical analyses in order that the data corresponded to the non-institutionalized civilian population. Continuous variables were represented by mean and standard deviation (SD), whereas categorical variables were expressed by counts and weighted percentages. One-way ANOVA test (for normally distributed continuous variables), Kruskal–Wallis H-test (for non-normally distributed continuous variables) and Chi-square test (for categorical variables) were utilized to measure differences among different groups.

Weighted logistic regression analysis was set up to estimate the relationship between n-3 PUFA poor/rich seafood consumption and gout. To further examine the covariable effect on this correlation, we employed Model 1 (unadjusted), Model 2 (age, gender, and race/ethnicity were adjusted), and Model 3 (all covariates in **Table 1** were adjusted). In addition, restricted cubic spline models were utilized to investigated their dose-response relationship in Model 3. Finally, we further explored the heterogenicity in different groups with interaction terms. $P < 0.05$ with effective confidence interval (CI) was of statistical significance. All analyses were constructed with R version 4.0.4[1] (The R foundation).

# Results

## Population characteristics between groups

As illustrated in **Figure 1**, a total of 12,505 subjects were enrolled in this study. **Table 1** summarized the population characteristics overall and between two groups (adults with/without gout). In the whole research population, the median age at baseline was 46.29 years, 48.33% were females and 51.17% were males. The overall prevalence of gout among US adults was 4.31%. Participants with gout were more likely be older, male, non-Hispanic White, obese, alcoholic, former smokers ($P < 0.01$). Additionally, they suffered from CKD, DM, hypertension, and had higher n-3 PUFA poor seafood intake ($P < 0.05$). There was no difference in income and education levels ($P > 0.05$).

## n-3 PUFA poor seafood intake is associated with higher risk of gout

Results of weighted logistic regression analysis for the association between n-3 PUFA poor/rich seafood consumption and gout are shown in **Table 2**. Weighted logistic regression analysis confirmed that higher n-3 PUFA poor seafood intake was associated with an increased risk of gout (Model 1, OR = 1.098, 95% CI: 1.053, 1.145, $P < 0.001$; Model 2, OR = 1.096, 95% CI: 1.048, 1.146, $P < 0.001$; Model 3, OR = 1.087, 95% CI: 1.039, 1.138, $P < 0.001$).

1 http://www.R-project.org

10.3389/fnut.2023.1075877

TABLE 1 Characteristics among adults with/without gout.

| Variable | Overall | Individuals without gout | Individuals with gout | *P*-value |
|---|---|---|---|---|
| Age, years, mean (SD) | 46.29 (0.31) | 45.80 (0.30) | 59.50 (0.70) | <0.001 |
| n-3 PUFA rich seafood intake, oz/day, mean (SD) | 0.19 (0.01) | 0.19 (0.01) | 0.29 (0.06) | 0.100 |
| n-3 PUFA poor seafood intake, oz/day, mean (SD) | 0.48 (0.02) | 0.46 (0.02) | 0.78 (0.13) | 0.020 |
| Gender, *n* (%) | | | | <0.001 |
| Female | 5,894 (48.83) | 5,777 (49.69) | 117 (25.57) | |
| Male | 6,611 (51.17) | 6,189 (50.31) | 422 (74.43) | |
| Race/ethnicity, *n* (%) | | | | <0.001 |
| Mexican American | 1,718 (7.21) | 1,687 (7.37) | 31 (2.71) | |
| Non-Hispanic Black | 2,370 (9.18) | 2,240 (9.13) | 130 (10.52) | |
| Non-Hispanic White | 6,071 (73.18) | 5,771 (72.95) | 300 (79.17) | |
| Other Hispanic | 1,202 (4.71) | 1,169 (4.81) | 33 (2.13) | |
| Other race (including multi-racial) | 1,144 (5.73) | 1,099 (5.74) | 45 (5.47) | |
| Income, *n* (%) | | | | 0.570 |
| Poor | 2,275 (11.75) | 2,178 (11.74) | 97 (12.22) | |
| Near poor | 4,841 (32.48) | 4,631 (32.58) | 210 (29.86) | |
| Not poor | 5,389 (55.76) | 5,157 (55.68) | 232 (57.91) | |
| Education, *n* (%) | | | | 0.790 |
| Less than high school | 2,259 (11.64) | 2,162 (11.63) | 97 (11.73) | |
| High school graduate/GED | 2,737 (20.39) | 2,602 (20.34) | 135 (21.88) | |
| Some college or AA degree | 3,951 (32.94) | 3,784 (32.90) | 167 (33.96) | |
| College graduate or more | 3,558 (35.03) | 3,418 (35.12) | 140 (32.43) | |
| BMI, *n* (%) | | | | <0.001 |
| Underweight (<18.5) | 168 (35.14) | 164 (1.31) | 4 (0.40) | |
| Normal (≥18.5 and ≤24.9) | 3,530 (1.28) | 3,442 (30.08) | 88 (13.97) | |
| Overweight (>24.9 and <30) | 4,247 (34.08) | 4,074 (34.14) | 173 (32.48) | |
| Obese (≥30) | 4,560 (29.5) | 4,286 (34.47) | 274 (53.15) | |
| CKD, *n* (%) | | | | <0.001 |
| No | 10,706 (88.19) | 10,372 (88.92) | 334 (68.65) | |
| Yes | 1,799 (11.81) | 1,594 (11.08) | 205 (31.35) | |
| DM, *n* (%) | | | | <0.001 |
| DM | 1,858 (11.5) | 1,660 (10.76) | 198 (31.50) | |
| IFG | 540 (4.5) | 503 (4.31) | 37 (9.47) | |
| IGT | 507 (3.75) | 478 (3.75) | 29 (3.89) | |
| No | 9,600 (80.25) | 9,325 (81.18) | 275 (55.13) | |
| Hypertension, *n* (%) | | | | <0.001 |
| No | 7,622 (64.65) | 7,514 (66.21) | 108 (22.76) | |
| Yes | 4,883 (35.35) | 4,452 (33.79) | 431 (77.24) | |
| Smoking status, *n* (%) | | | | <0.001 |
| Former | 3,219 (26.35) | 2,990 (25.70) | 229 (43.73) | |
| Never | 6,417 (52.71) | 6,204 (53.13) | 213 (41.34) | |
| Current | 2,869 (20.95) | 2,772 (21.17) | 97 (14.92) | |
| Drinking status, *n* (%) | | | | <0.001 |
| Mild | 6,166 (49.48) | 5,844 (49.10) | 322 (59.51) | |
| Moderate | 2,797 (23.3) | 2,710 (23.61) | 87 (14.97) | |
| Heavy | 3,542 (27.22) | 3,412 (27.28) | 130 (25.53) | |

SD, standard deviation; n-3 PUFA, omega-3 polyunsaturated fatty acid; oz, ounce; GED, General Education Development; AA degree, Associate of Arts; BMI, body mass index; HUA, hyperuricemia; CKD, chronic kidney disease; DM, diabetes mellitus; IFG, impaired fasting glucose; IGT, impaired glucose tolerance.

TABLE 2 Association between n-3 PUFA rich/poor seafood intake and gout.

| Variable | OR (95% CI), *P*-value | | |
|---|---|---|---|
| | Model 1 | Model 2 | Model 3 |
| n-3 PUFA rich seafood intake | 1.112 (1.017, 1.216), 0.019 | 1.045 (0.947, 1.153), 0.380 | 1.070 (0.969, 1.183), 0.180 |
| n-3 PUFA poor seafood intake | 1.098 (1.053, 1.145), <0.001 | 1.096 (1.048, 1.146), <0.001 | 1.087 (1.039, 1.138), <0.001 |

Model 1: no adjustment.
Model 2: adjusted for age, gender, and race/ethnicity.
Model 3: adjusted for age, gender, race/ethnicity, income, education, BMI, CKD, DM, hypertension, smoking status, and drinking status.
OR, odds ratio; CI, confidence interval; n-3 PUFA, omega-3 polyunsaturated fatty acid; BMI, body mass index; CKD, chronic kidney disease; DM, diabetes mellitus.

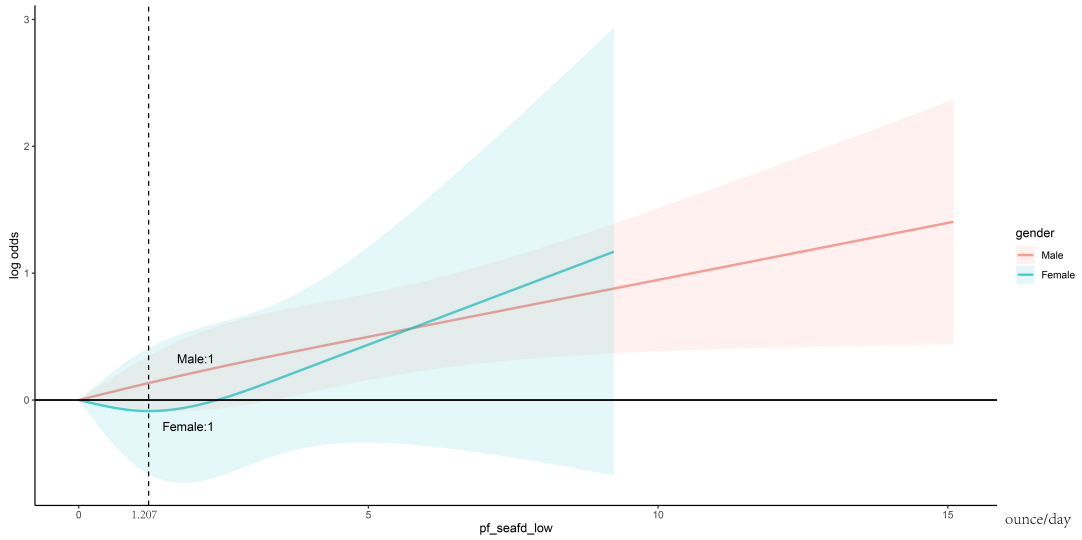


FIGURE 2
The dose-response relationship between n-3 PUFA poor seafood intake and gout. Adjusted for age, gender, race/ethnicity, income, education, BMI, CKD, DM, hypertension, smoking status, and drinking status. Solid lines hazard ratio, blue or pink area 95% confidence interval. n-3 PUFA, omega-3 polyunsaturated fatty acid; BMI, body mass index; CKD, chronic kidney disease; DM, diabetes mellitus.

In model 3, which adjusted for all confounding variables, the results still revealed that each unit of increase of n-3 PUFA poor seafood intake was associated with an 8.7% increased risk of gout. With respect to n-3 PUFA rich seafood intake, the connection with gout was only significant in model 1(OR = 1.112, 95% CI: 1.017, 1.216, $P = 0.019$), but become non-significant after stepwise adjusting for confounding variables (Models 2 and 3). In dose-response relationships, n-3 PUFA rich seafood consumption was non-linearly related with gout in the female group. We found an inverted L-shaped association. The prevalence of gout reached a plateau when the n-3 PUFA poor seafood intake was lower than 1.207 ounce/day in the female group. Figure 2 depicts the dose response relationship. We also conducted subgroup analyses stratified by age, gender, race/ethnicity, income, education, BMI, CKD, DM, hypertension, smoking, and drinking status to evaluate the association between n-3 PUFA rich seafood intake and gout (Figure 3). This association was similar among participants aged >37 and <56 years old. Relatively stronger relationships were also observed among male, non-Hispanic White, other race and patients with underweight, normal weight, overweight, IFG, non-DM, mild drinking, and moderate drinking. Additionally, statistically significant ORs were only present in people without CKD, hypertension and with a lower household PIR and current smoking. Besides, we also explored the heterogenicity among each subgroup with interaction terms and no significant difference was revealed among age, gender, race/ethnicity, income, education, CKD, DM, hypertension, smoking status, and drinking status (*P* for interaction >0.05 for all), revealing that the magnitude of this relationship was the same for the subjects separated into different subgroups.

## Discussion

In this nationwide cross-sectional study with 12,505 adults, a significant positive connection of n-3 PUFA poor seafood intake with gout was uncovered, revealing that higher consumption of n-3 PUFA poor seafood may contribute to an increased risk of gout. This connection remained statistically significant after we adjusted for all confounders, including age, gender, race/ethnicity, income, education, BMI, CKD, DM, hypertension, smoking status, and drinking status. Correlated subgroup analyses stratified by different variables revealed that this positive connection was not influenced, suggesting that this connection could be appropriated for different population.

It was worth noting that there was a lack of research on the effect of n-3 PUFA poor/rich seafood consumption on gout. It is well established that purine-rich foods consumption, which comprised most seafood, brings about an increased risk of gout (17). Interestingly, our study demonstrated that only n-3 PUFA

| Subgroup | OR_95%CI | P_value | P for interaction |
|---|---|---|---|
| Age | | | 0.322 |
| Tertile1 | 0.978(0.782, 1.224) | 0.844 | |
| Tertile2 | 1.117(1.012, 1.234) | 0.029 | |
| Tertile3 | 1.073(0.955,1.206) | 0.229 | |
| Gender | | | 0.855 |
| Female | 1.079(0.908, 1.281) | 0.381 | |
| Male | 1.101(1.021,1.186) | 0.013 | |
| Race | | | 0.415 |
| Mexican American | 1.021(0.773, 1.348) | 0.881 | |
| Non-Hispanic Black | 1.087(0.991, 1.192) | 0.075 | |
| Non-Hispanic White | 1.110(1.006,1.224) | 0.039 | |
| Other Hispanic | 0.820(0.580, 1.160) | 0.256 | |
| Other Race - Including Multi-Racial | 1.153(1.047, 1.270) | 0.005 | |
| Income | | | 0.322 |
| Not poor | 1.123(0.989, 1.275) | 0.073 | |
| Near poor | 1.033(0.942,1.132) | 0.485 | |
| Poor | 1.174(1.021, 1.349) | 0.025 | |
| Education | | | 0.491 |
| Less than high scool | 1.096(0.977, 1.229) | 0.115 | |
| High School graduate/GED | 1.050(0.951,1.160) | 0.324 | |
| Some College or AA degree | 1.058(0.921,1.216) | 0.418 | |
| College graduate or more | 1.166(0.997, 1.363) | 0.054 | |
| BMI | | | 0.005 |
| underweight | 0.002(0.001,0.004) | 0.006 | |
| normal | 1.208(1.066, 1.369) | 0.004 | |
| overweight | 1.175(1.080,1.278) | <0.001 | |
| obese | 0.982(0.878, 1.097) | 0.738 | |
| CKD | | | 0.113 |
| Yes | 0.976(0.859, 1.109) | 0.704 | |
| No | 1.128(1.037,1.227) | 0.006 | |
| DM | | | 0.292 |
| Yes | 1.038(0.923, 1.167) | 0.525 | |
| IFG | 1.204(1.030, 1.407) | 0.020 | |
| IGT | 0.912(0.684, 1.215) | 0.522 | |
| No | 1.132(1.042,1.230) | 0.004 | |
| Hypertension | | | 0.164 |
| Yes | 1.056(0.976,1.144) | 0.173 | |
| No | 1.168(1.055, 1.294) | 0.003 | |
| Smoking | | | 0.678 |
| never | 1.053(0.945, 1.174) | 0.339 | |
| former | 1.132(0.964,1.328) | 0.128 | |
| now | 1.095(1.012,1.186) | 0.025 | |
| Drinking | | | 0.129 |
| mild | 1.140(1.024, 1.270) | 0.018 | |
| moderate | 1.164(1.041, 1.301) | 0.008 | |
| heavy | 0.980(0.828,1.160) | 0.81 | |

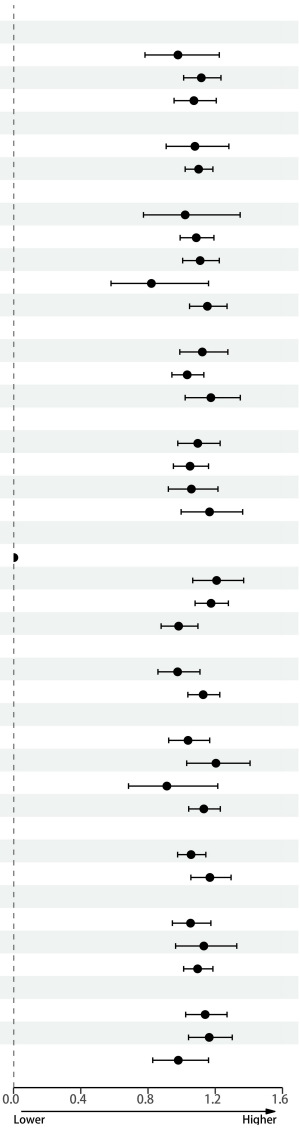


FIGURE 3
Subgroup analysis between n-3 PUFA poor seafood intake and gout. The subgroup analysis was adjusted for age, gender, race/ethnicity, income, education, BMI, CKD, DM, hypertension, smoking status, and drinking status. Age (years old), Tertile 1, 20–37, Tertile 2, 37–56, Tertile 3, 56–80; n-3 PUFA, omega-3 polyunsaturated fatty acid; OR, odds ratio; CI, confidence interval; GED, General Education Development; AA degree, Associate of Arts; BMI, body mass index; CKD, chronic kidney disease; DM, diabetes mellitus; IFG, impaired fasting glucose; IGT, impaired glucose tolerance.

poor seafood consumption was associated with increased risk of gout with statistical significance. The results of our study also provided a proof-of-concept regarding the potential for n-3 PUFA rich seafood to counteract harmful effects of purines in relation to gout. Emerging evidence has shown that n-3 PUFAs could exert protective anti-inflammatory effects. A meta-analysis revealed that supplementation of n-3 PUFAs in adults can improve inflammatory biomarkers, such as serum C-reactive protein, tumor necrosis factor α (TNF-α), and interleukin 6 (IL-6) (31).

Several hypotheses could be put forward to explain the anti-inflammatory effects of n-3 PUFAs. First, n-3 PUFAs may involve in the regulation of immunological and inflammatory responses *via* the gut microbiome (32–34). Gut microbiota can influence the metabolism and absorption of n-3 PUFAs, in the meantime, n-3 PUFAs can also affect the diversity and abundance of the gut microbiome. For example, n-3 PUFAs possibly suppressed the *Firmicutes/Bacteroidetes* ratio and the levels of *Coprococcus* and *Faecalibacterium* and increased the abundance of butyrate-producing bacterial genera, thus further reducing the inflammatory processes (35–37).

Second, n-3 PUFAs are important mediators in membrane phospholipid fatty acid composition of inflammatory cells (38). n-3 PUFAs could vary the composition of membrane phospholipid fatty acid *via* a variety of general mechanisms, resulting in affecting inflammatory cell function. To begin with, the physical properties of the membrane, such as membrane fluidity and raft structure could be altered by n-3 PUFAs (38). Then, n-3 PUFAs could exert influences on cell signaling pathways. Certain n-3 PUFAs have been shown to have anti-inflammatory effects through inhibition of the principal inflammatory cytokine *via* extracellular inflammatory stimuli (39) and G-protein coupled

receptors (40). An *in vivo* experiment revealed that peroxisome proliferator-activated receptor γ, an anti-inflammatory factor, was induced by n-3 PUFAs in dendritic cells (41). Furthermore, n-3 PUFAs could alter the production in the pattern of the lipid mediators. Animal studies have shown that n-3 PUFAs may modify the fatty acid of eicosanoid, a key mediators of inflammation, resulting in producing several anti-inflammatory and inflammation resolving mediators, such as resolvins and protectins (39). Finally, with respect to T cell signaling, n-3 PUFAs were found to disturb membrane-cytoskeletal structure and function in CD4+ T lymphocytes (33).

Third, n-3 PUFAs may exert anti-inflammatory effects in macrophages. The balance between pro- and anti-inflammation is coordinated by macrophages. Previous animal studies have demonstrated that adding n-3 PUFA to the diet induced a decrease in macrophages (40, 42). In studies of mechanisms, the anti-inflammatory function by n-3 PUFAs has been proved through the free fatty acid receptor 4 protein in macrophages resulting in suppressing activity of the NF-κB complex (42). Otherwise, *in vivo* findings have been supported that n-3 PUFAs regulated inflammatory signaling in macrophages *via* the autophagic receptor SQSTM1/p62-bodies and NFE2L2 (43).

The major strengths of this population-based study are using a nationally representative sample, which facilitates the finding to be universal to a broader population. Nevertheless, several limitations cannot be ignored. First, there were only two 24-h dietary recalls in NHANES rather than three 24-h dietary recalls (two weekdays and one Friday), where recall bias is inevitable.

To minimize the influence of two-time recall bias, the NHANES design utilized sampling weight and multiple-pass method to ensure precise of dietary intake and subjects who had both two valid 24-h dietary recalls were included in this study. Second, we were unable to consider more purine-rich foods except alcohol consumption, which may lead to confounding bias. Third, cross-section design cannot draw causal relationship.

## Conclusion

In summary, our results suggest that n-3 PUFA poor seafood consumption is associated with higher risk of gout, whereas n-3 PUFA rich seafood is not and also provide a proof-of-concept regarding the potential for n-3 PUFA rich seafood to counteract harmful effects of purines in relation to gout. A dose-response analysis showed that there was a non-linear relationship between n-3 PUFA rich seafood intake and the risk of gout in the female group. Given this cross-section design, more well-designed prospective studies are warranted to validate the causal relationship between n-3 PUFA poor/rich seafood and gout.

## Data availability statement

The original contributions presented in this study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

## Author contributions

GZ and JW: conceptualization. GZ and DY: methodology. GZ, DY, and JW: software. GZ, LY, and JW: formal analysis. GZ, DY, LY, HS, JL, YW, and ZJ: data collection. GZ: writing—original draft preparation. JW: writing—review and editing and supervision. All authors read and agreed to the published version of the manuscript.

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## Conflict of interest

The 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.

## Publisher's note



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# A commercial blend of macroalgae and microalgae promotes digestibility, growth performance, and muscle nutritional value of European seabass (*Dicentrarchus labrax* L.) juveniles

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Algae can leverage aquaculture sustainability and improve the nutritional and functional value of fish for human consumption, but may pose challenges to carnivorous fish. This study aimed to evaluate the potential of a commercial blend of macroalgae (*Ulva* sp. and *Gracilaria gracilis*) and microalgae (*Chlorella vulgaris* and *Nannochloropsis oceanica*) in a plant-based diet up to 6% (dry matter basis) on digestibility, gut integrity, nutrient utilization, growth performance, and muscle nutritional value of European seabass juveniles. Fish (11.3 ± 2.70 g) were fed with isoproteic, isolipidic, and isoenergetic diets: (i) a commercial-type plant-based diet with moderate fishmeal (125 g $kg^{-1}$ DM basis) and without algae blend (control diet; Algae0), (ii) the control diet with 2% algae blend (Algae2), (iii) the control diet with 4% algae blend (Algae4), and (iv) the control diet with 6% algae blend (Algae6) for 12 weeks. The digestibility of experimental diets was assessed in a parallel study after 20 days. Results showed that most nutrients and energy apparent digestibility coefficients were promoted by algae blend supplementation, with a concomitant increase in lipid and energy retention efficiencies. Growth performance was significantly promoted by the algae blend, the final body weight of fish fed Algae6 being 70% higher than that of fish fed Algae0 after 12 weeks, reflecting up to 20% higher feed intake of algae-fed fish and the enhanced anterior intestinal absorption area (up to 45%). Whole-body and muscle lipid contents were increased with dietary algae supplementation levels by up to 1.79 and 1.74 folds in Algae 6 compared to Algae0, respectively. Even though the proportion of polyunsaturated fatty acids was reduced, the content of EPA and DHA in the muscle of algae-fed fish increased by nearly 43% compared to Algae0. The skin and filet color of juvenile European seabass were significantly affected by the dietary inclusion of the algae blend, but changes were small

in the case of muscle, meeting the preference of consumers. Overall results highlight the beneficial effects of the commercial algae blend (Algaessence®) supplementation in plant-based diets for European seabass juveniles, but feeding trials up to commercial-size fish are needed to fully assess its potential.

KEYWORDS

**algae blend, digestibility, growth performance, gut integrity, functional value, microalgae, muscle quality, seaweed**

## 1. Introduction

The quest for healthy and nutritious food is one of the main concerns of the Food and Agriculture Organization (FAO) of the United Nations to address food security and the growing demand for animal protein sources by the world's growing population, expected to reach 9.7 billion by 2050 (1). According to the latest report, severe food insecurity in 2021 increased compared to 2020 (2), mainly due to the long-lasting effects of the COVID-19 pandemic. A trend that may continue as a result of the ongoing war and the political, economic, and financial uncertainty. Indeed, FAO anticipates that 670 million people may suffer from hunger by 2030, a figure similar to 2015 when the Sustainable Development Goals of the 2030 Agenda was launched (2). It is thus of paramount importance to produce high-quality foods to address food insecurity.

Aquaculture, the fastest-growing sector of the food industry, has the potential to contribute to food security and meet the nutritional requirements of the world's growing population (3). To address the growing demand for aquafeeds and the limited supply of fishmeal and fish oil, fewer marine sources and more plant sources are currently used in nutrient-based formulations that not only meet fish nutrient and energy requirements but also attend to their nutraceutical and functional properties (4, 5). These modern formulations of aquafeed pose challenges to fish, in particular to carnivorous species such as European seabass (*Dicentrarchus labrax* L.), a marine species of high commercial importance in European aquaculture, particularly in the Mediterranean region (6). Compared to marine sources, land plant sources have lower digestibility, are deficient in essential amino acids, such as lysine and methionine, and have a lipid profile rich in *n*-6 and completely lacking *n*-3 long-chain polyunsaturated fatty acids (LC-PUFA) (7). The search for alternative, locally produced, and more sustainable ingredients to be included in modern aquafeed formulations is of paramount importance to reduce or replace traditional marine sources, as well as plant ingredients with a high environmental footprint, nutrient imbalance, or antinutritional compounds or that are used for terrestrial animal feeding or human consumption (8). By addressing aquaculture sustainability and improving the nutritional and functional value of fish for human consumption, the search for alternative aquafeed ingredients fits into the One Health concept and the goal to achieve the best health outcomes for fish, consumers, and the environment.

Most research on alternative aquafeed ingredients has focused on alternative plants, rendered animal and aquaculture by-products, insects, single-cell organisms, and algae (8–10). Algae, including microalgae and macroalgae, are of particular interest due to their high growth rates and biomass productivity, low environmental footprint, and non-competition with other cultures for arable land, being even able to grow in waste water (11). In addition, algae are valuable sources of macro- and micro-nutrients and bioactive compounds (12–14), with levels varying with species and within species with abiotic and biotic growth conditions (15, 16). Microalgae contain all essential amino acids, and some species are rich sources of protein and lipids, with marine species being particularly rich in *n*-3 LC-PUFA, such as eicosapentaenoic acid (EPA; C20:5 *n*-3) and docosahexaenoic acid (DHA; C22:6 *n*-3) (17–19) with important health-promoting properties (20). On the contrary, macroalgae species have lower protein content with a less balanced amino acids profile, in particular, brown macroalgae, and are poor sources of lipids, although marine species have a fairly high proportion of LC-PUFA (16, 21). Both microalgae and macroalgae are good sources of complex polysaccharides, pigments, and organic minerals with potential health-promoting effects such as prebiotic, immunomodulatory, and antioxidant activities (12, 14, 22, 23). Although algae lack lignin and are poor in hemicellulose (24, 25), the complex structural polysaccharides of eukaryotic microalgae and macroalgae may reduce the digestibility and availability of macro- and micronutrients for carnivorous fish (16, 18, 26). In face of their nutritional value, the potential of micro- and macroalgae as alternative aquafeeds has been suggested for fingerlings and fish diets, including the replacement of traditional (fishmeal) and current (plant-based) protein sources (10, 18, 25) and lipid sources such as fish oil (27). In addition, algae are sustainable and valuable sources of bioactive compounds, in particular, *n*-3 LC-PUFA, that are essential for aquafeed formulations (12, 28). Although their high production costs are still a bottleneck for algae use in fish diets as ingredients, it is anticipated that in the near future this limitation can be overcome (25).

In this context, several studies have evaluated the inclusion of individual species of microalgae or macroalgae in carnivorous fish diets, namely Atlantic salmon (*Salmo salar*) (29), European seabass (30–32), turbot (*Scophthalmus maximus*) (33), barramundi (*Lates calcarifer*) (34), meager (*Argyrosomus regius*) (35), Persian sturgeon (*Acipenser persicus*) (36), and Senegalese sole (*Solea senegalensis*) (37). In most studies, growth or feed efficiency was not impaired at dietary inclusion levels up to 10% (dry matter, DM, basis), but in some cases, decreased nutrient and energy digestibility was

reported (13, 26). At higher inclusion levels, growth performance, feed utilization, and digestibility of diets were reduced (38–41). Available data support species-specific and dose-dependent effects of dietary algae inclusion (42) and highlight the need for further studies.

*Chlorella* spp. and *Nannochloropsis* spp. are among the most produced microalgae species in Europe (82 and 21 tons DM $year^{-1}$, respectively) (43). *Chlorella vulgaris* is a rich source of protein (51.5–67.7% DM basis) (18), with a high content of essential amino acids, particularly arginine, lysine, and leucine (44), while *N. oceanica* is an oleaginous microalga with the ability to accumulate EPA, a health-promoting *n*-3 LC-PUFA (20). Although produced on a small scale at the European level, the production of macroalgae species native to the Mediterranean, such as *Ulva* spp. and *Gracilaria* spp., has a considerable expression in Portugal (43). These macroalgae species are rich sources of polysaccharides (30–75% DM basis) (16), including sulfated polysaccharides, such as ulvan in *Ulva* spp. and carrageenan and agar in *Gracilaria* spp. (45), with prebiotic, immunomodulatory, and antioxidant properties (46–48).

Although the combination of algae species has been suggested to improve the nutritional and functional value of diets (49), few studies have evaluated the supplementation of mixtures of inclusion of microalgae or macroalgae species in European seabass diets (50–53), and only one study has evaluated the combination of one micro- and one macroalgae species in an *in vivo* trial with *D. labrax* (54). We hypothesize that the combination of several species of micro- and macroalgae can exert strong synergistic effects and improve the nutritional and functional value of modern plant source-based aquafeeds. Thus, the present study aimed to evaluate the effects of dietary inclusion of an algae blend composed of two macroalgae (*Ulva* sp. and *Gracilaria gracilis*) and two microalgae (*Chlorella vulgaris* and *Nannochloropsis oceanica*) on digestibility, gut integrity, nutrient utilization, growth performance, and muscle quality of European seabass juveniles.

# 2. Materials and methods

Two trials were conducted at the Fish Culture Experimental Unit of CIIMAR (Matosinhos, Portugal) to evaluate the potential of the algae blend as a novel feed for European seabass juveniles: a digestibility trial and a growth trial. All procedures with animals were reviewed and approved by the Animal Welfare and Ethics Body of CIIMAR (ORBEA-CIIMAR 06-2016), licenced by the Portuguese Veterynary Authority (1005/92, DGAV-Portugal), and carried out by trained researchers accredited in laboratory animal science following FELASA C recommendations. The experiments were conducted in strict compliance with European Union guidelines on the protection of animals for scientific purposes (Directive 2010/63/EU).

## 2.1. Fish

European seabass (*D. labrax*) juveniles were obtained from a commercial fish farm (Acuinuga, S.L., La Coruña, Spain), transported to CIIMAR facilities, and kept in quarantine for 3 weeks. During this period, the fish were fed a commercial diet (49% crude protein, CP, and 20% ether extract, EE, DM basis; AQUASOJA, Sorgal, S.A., Ovar, Portugal). After acclimation, the fish were fasted for 24 h, anesthetized (60 μl $L^{-1}$ of 2-phenoxyethanol, Sigma-Aldrich, St. Louis, MO, USA), and individually weighted (g) and measured (total length, cm). Homogeneous groups of fish were then distributed among the tanks used for the digestibility and growth trials.

## 2.2. Algae blend and experimental diets

The algae blend used in this study, composed of two macroalgae species (*Ulva* sp. and *G. gracilis*) and two microalgae species (*C. vulgaris* and *N. oceanica*), is a commercial product (Algaessence® feed) produced by ALGAplus (Ílhavo, Portugal) and Allmicroalgae (Pataias, Portugal). The blend was supplied as a spray-dried power in sealed bags protected from light.

Four isoproteic (527 g $kg^{-1}$ DM basis), isolipidic (153 g $kg^{-1}$ DM basis), and isoenergetic (21.7 MJ $kg^{-1}$ DM basis) diets were formulated according to the nutritional requirements of European seabass juveniles (55) and considering current trends in commercial aquafeeds of high vegetable protein sources (c.a., 700 g $kg^{-1}$ DM basis) and moderate fishmeal (125 g $kg^{-1}$ DM basis) inclusion level. The algae blend was included in the experimental diets at expense of wheat gluten and whole peas, with levels of fishmeal and fish oil held constant. The experimental diets were as follows: i) a commercial plant protein-based diet without algae blend inclusion (control diet; Algae0), ii) the control diet with 2% algae blend inclusion (Algae2), iii) the control diet with 4% algae blend inclusion (Algae4), and iv) the control diet with 6% algae blend inclusion (Algae6). Yttrium oxide ($Y_2O_3$, 0.2 g $kg^{-1}$, DM basis) was included in all diets as an inert marker for determining the apparent digestibility coefficients. Diets were manufactured and extruded by SPAROS Lda. (Olhão, Portugal), using a pilot-scale twin-screw extruder (CLEXTRAL BC45, Firminy, France). The pellets (2.0 mm) were dried in a convection oven (OP 750-UF, LTE Scientific, Oldham, UK), and the fish oil was added by vacuum coating (Pegasus PG-10VCLAB, DINNISSEN, Sevenum, Netherlands). The diets were stored at 4°C until use. The ingredients and chemical composition of the experimental diets are shown in Table 1 and Supplementary Table S1.

### 2.2.1. Proximate analysis

The algae blend and the ground (1-mm) experimental diets were homogenized, and their proximate composition was analyzed in duplicate according to official methods (56). Samples were analyzed for DM (ID 934.01), ash (ID 942.05), and nitrogen (N) (ID 990.03) contents. Crude protein was calculated as N x 6.25 (ID 990.03). Gross energy (GE) was determined using an adiabatic bomb calorimeter (Werke C2000, IKA, Staufen, Germany). The starch content was analyzed in 0.5-mm ground samples (57). Crude fiber (CF; ID 962.09), neutral detergent fiber (NDF), and acid detergent fiber (ADF) (58, 59) of the algae blend and diets were also determined. Due to the small size of the microalgae species present in the blend (<25 μm diameter), the filtration step to determine the fiber content (CF, NDF, and ADF) was modified by replacing the P2 crucibles (porosity 40–100 μm) with glass

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TABLE 1 Ingredient composition (g $kg^{-1}$, as is) and proximate composition, essential amino acids, and selected fatty acids content (g $kg^{-1}$, dry matter) of the algae blend and experimental diets.

| | Algae blend | Diets | | | |
|---|---|---|---|---|---|
| | | Algae0 | Algae2 | Algae4 | Algae6 |
| **Ingredient composition** | | | | | |
| Norwegian fishmeal LT 70[a] | | 125 | 125 | 125 | 125 |
| Soy protein concentrate[b] | | 300 | 300 | 300 | 300 |
| Wheat gluten[c] | | 110 | 105 | 101 | 96.0 |
| Corn gluten[d] | | 125 | 125 | 125 | 125 |
| Soybean meal 48[e] | | 100 | 100 | 100 | 100 |
| Whole peas | | 82.8 | 68.8 | 52.8 | 37.8 |
| Fish oil[f] | | 132 | 131 | 131 | 131 |
| Vitamin and mineral premix[g] | | 5.00 | 5.00 | 5.00 | 5.00 |
| Monocalcium phosphate[h] | | 20.0 | 20.0 | 20.0 | 20.0 |
| Yttrium oxide | | 0.200 | 0.200 | 0.200 | 0.200 |
| Algae blend[i] | | - | 20.0 | 40.0 | 60.0 |
| **Proximate composition** | | | | | |
| Ash | 251 | 74.2 | 78.6 | 83.0 | 86.4 |
| Crude protein | 347 | 526 | 529 | 525 | 530 |
| Total lipids | 59.5 | 128 | 130 | 121 | 129 |
| Crude fiber | 24.5 | 29.0 | 28.1 | 27.1 | 25.3 |
| Neutral detergent fiber | 126 | 120 | 131 | 127 | 121 |
| Acid detergent fiber | 53.2 | 60.4 | 60.4 | 61.6 | 62.0 |
| Starch | 21.7 | 55.7 | 50.6 | 45.8 | 38.7 |
| Gross energy (MJ $kg^{-1}$) | 16.5 | 21.8 | 21.5 | 21.7 | 21.8 |
| Essential amino acids | 187 | 261 | 254 | 249 | 250 |
| Arginine | 28.7 | 41.7 | 40.5 | 38.9 | 39.5 |
| Histidine | 6.08 | 13.3 | 12.4 | 11.8 | 11.8 |
| Isoleucine | 15.9 | 23.4 | 23.1 | 22.8 | 23.3 |
| Leucine | 27.3 | 41.0 | 41.6 | 40.8 | 40.7 |
| Lysine | 30.2 | 29.0 | 31.3 | 31.4 | 33.1 |
| Methionine | 7.02 | 10.2 | 8.87 | 9.02 | 8.91 |
| Methionine + Cystine | 8.62 | 13.4 | 12.0 | 11.7 | 11.5 |
| Phenylalanine | 18.9 | 29.4 | 26.4 | 26.1 | 24.9 |
| Phenylalanine + Tyrosine | 35.4 | 54.3 | 49.7 | 48.9 | 46.2 |
| Threonine | 15.4 | 21.1 | 20.7 | 20.4 | 20.3 |
| Valine | 19.1 | 24.1 | 22.9 | 23.0 | 23.2 |
| Fatty acids | 54.8 | 159 | 161 | 162 | 162 |
| C16:0 | 13.5 | 31.6 | 31.5 | 31.4 | 31.2 |
| C18:0 | 0.536 | 6.13 | 6.05 | 6.16 | 6.08 |
| C16:1 *n*-7 | 7.39 | 12.4 | 12.5 | 12.5 | 12.5 |
| C18:1 *n*-9 | 2.13 | 17.9 | 17.7 | 17.5 | 17.2 |
| C18:2 *n*-6 | 4.00 | 10.7 | 10.6 | 10.3 | 10.1 |

*(Continued)*

TABLE 1 (Continued)

| | Algae blend | Diets | | | |
|---|---|---|---|---|---|
| | | Algae0 | Algae2 | Algae4 | Algae6 |
| C18:3 *n*-3 | 5.52 | 1.81 | 1.92 | 2.00 | 2.09 |
| C20:5 *n*-3 (EPA) | 5.93 | 21.1 | 22.3 | 23.1 | 23.6 |
| C22:6 *n*-3 (DHA) | ND | 15.3 | 15.2 | 15.3 | 15.2 |
| EPA + DHA | 5.93 | 36.4 | 37.5 | 38.4 | 38.8 |

Algae0, commercial-based diet without algae blend inclusion (control diet); Algae2, control diet with 2% algae blend inclusion; Algae4, control diet with 4% algae blend inclusion; Algae6, control diet with 6% algae blend inclusion; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; ND, not detected.
[a]NORVIK LT 70: 70.6% crude protein (CP), 5.8% ether extract (EE) (Sopropêche, Wimille, France).
[b]Soycomil® -P: 63% CP, 0.8% EE (ADM, Amsterdam, The Netherlands).
[c]Wheat gluten meal: 10.2% CP, 1.2% EE (Casa Lanchinha, Alhos Vedros, Portugal).
[d]Corn gluten meal: 61% CP, 6% EE (COPAM, São João da Talha, Portugal).
[e]Solvent extracted dehulled soybean meal: 47% CP, 1.6% EE (CARGILL, Barcelona, Spain).
[f]Fish oil (Sopropêche, Wimille, France).
[g]Vitamins (IU or mg/kg diet): 100 mg DL-alpha tocopherol acetate, 25 mg sodium menadione bisulfate, 20,000 IU retinyl acetate, 2.000 IU DL-cholecalciferol, 30 mg thiamin, 30 mg riboflavin, 20 mg pyridoxine, 0.1 mg cyanocobalamin, 200 mg nicotinic acid, 15 mg folic acid, 500 mg ascorbic acid; 500 mg inositol, 3 mg biotin, 100 mg calcium pantothenate, 1,000 mg choline chloride, 500 mg betaine; Minerals (g or mg/kg diet): 9 mg copper sulfate, 6 mg ferric sulfate, 0.5 mg potassium iodide, 9.6 mg manganese oxide, 0.01 mg sodium selenite, 7.5 mg zinc sulfate, 400 mg sodium chloride; excipient: wheat middling's (Wisium, Cantanhede, Portugal).
[h]Monocalcium phosphate: 21.8% P, 18.4% Ca (Fosfitalia, Ravenna, Italy).
[i]Algaessence® feed (Allmicroalgae/ALGAplus, Portugal).

microfiber filter (Whatman GF/A, 1.6 μm porosity, Merck KGaA, Darmstadt, Germany). Fiber fractions were expressed exclusive of residual ash. Analyses were run in duplicate.

### 2.2.2. Lipids and fatty acids analyses

The total lipids were quantified following the method of Folch et al. (60), modified by using dichloromethane:methanol (2:1) instead of trichloromethane:methanol (2:1). The fatty acids were transesterified to fatty acid methyl esters by acid-catalyzed methylation (61). Non-adecanoic acid (C19:0, Matreya LLC, State College, PA, USA) was added as an internal standard. Fatty acid methyl esters were analyzed by gas chromatography, using a Shimadzu GC-2010 Plus gas chromatograph (Shimadzu Europe GmbH, Duisburg, Germany) equipped with a capillary column (Omegawax 250, 30 m × 0.25 mm × 0.25 μm; Supelco, Bellefonte, PA, USA) and a flame-ionization detector. The carrier gas was helium at 1.30 ml $min^{-1}$, with a split ratio of 1:100, and the injection volume was 1.0 μl. The initial column temperature of 150°C was held for 7 min, increased at 3°C $min^{-1}$ to 170°C and held for 25 min, and then increased at 3°C $min^{-1}$ to 220°C and held for 30 min. The injector and detector temperatures were 250 and 260°C, respectively. Fatty acids were identified by comparing retention times with those of commercially available standards (Supelco 37 Component FAME Mix, BAME Mix, PUFA No.1, PUFA No.2, PUFA No.3, Sigma-Aldrich Co. LLC; GLC-110 Mixture, Matreya LLC) and quantified by using the internal standard (C19:0). Analyses were run in duplicate.

### 2.2.3. Amino acids analysis

The amino acid content of the algae blend and experimental diets were determined after hydrolysis with 6 M HCl at 116°C, for 48 h, followed by pre-column derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (Waters AccQ Fluor Reagent; Waters, Milford, MA, USA) as described by Aragão et al. (62), using norvaline (Waters) as an internal standard. Amino acids were analyzed by ultra-high-performance liquid chromatography on a Waters reverse phase amino acid analysis system and identified by comparison of retention times of commercial standard mixtures (Waters) and pure standards (Sigma-Aldrich Co. LLC). Data were acquired and analyzed using the EMPOWER software (Waters). The analysis was run in duplicate.

### 2.2.4. Element analysis

Macro and trace elements of algae blend and diets were determined after mineralization in a Milestone (Sorisole, Italy) MLS 1200 Mega high-performance microwave digestion unit (63). Samples were analyzed by inductively coupled plasma-mass spectrometry (ICP-MS; Thermo Fisher Scientific iCAP Q ICP-MS instrument, Waltham, MA, USA) and flame atomic absorption spectrometry (FAAS; PerkinElmer AAnalyst 200 FAAS instrument, Waltham). The calibration standards for FAAS were prepared from single-element standard stock solutions (Fluka, Buchs, Switzerland) by appropriate dilution with $HNO_3$ 0.2% (v/v). For ICP-MS determinations, internal standards and tuning solutions were prepared by appropriate dilution of the following solutions: periodic table mix 3 for ICP-MS (TraceCERT®, Sigma-Aldrich) containing 10 mg $L^{-1}$ of 16 elements (Sc, Y, La, Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb, and Lu in 5% $HNO_3$) and a custom solution (SCP Science, Quebec, Canada) with 1 mg $L^{-1}$ of Ba, Bi, Ce, Co, In, Li, and U in a 5% $HNO_3$ + 0.5% HCl, respectively. The iodine content of the algae blend and diets were also determined. After alkaline extraction with tetramethylammonium hydroxide (TMAH, Sigma-Aldrich Co. LLC) at a high temperature (90 ± 3 °C) for 3 h, iodine was analyzed by ICP-MS, and the concentration was calculated based on external calibration with iodine standards prepared in 0.5% (v/v) TMAH (64). The analyses were carried out in triplicate.

## 2.3. Digestibility trial

From an initial lot of European seabass juveniles, six homogeneous groups of 30 fish (14.1 ± 6.36 g of body weight, BW)

were randomly allocated to 50 L fiberglass tanks with individual feces sedimentation columns, in a Guelph system as described by Cho and Slinger (65). Fish were adapted to the new conditions (21.5 ± 0.27°C of water temperature, 35.7 ± 1.15‰ of salinity, 2 L $min^{-1}$ of flow rate, and 12 h of light/dark photoperiod) for 19 days. During this period, fish were fed a commercial diet (49% CP and 20% EE, DM basis; AQUASOJA, Sorgal, S.A.). After acclimatization, the digestibility of the experimental diets was assessed in two runs of 20 days; 7 days for adaptation to each experimental diet and 13 days for total feces collection. The experimental diets were hand-fed until apparent satiation two times a day (9:00 and 17:00 h), 7 days a week. During the feces collection period, the tank and the sedimentation column were thoroughly cleaned after 30 min of feeding to ensure that all uneaten feed was removed. Feces were collected from the sedimentation column two times a day, before feeding, centrifuged at 3,000 x *g* for 5 min at 4°C to eliminate the excess water, and stored at −20°C until further analysis. At the end of the first run, the fish were fasted for 24 h for gut evacuation. After this period, the second run of the digestibility trial began. Diets were randomly distributed by the tanks in the first run and caution was taken to ensure that diets were not allocated to the same tank in the second run. By the end of the digestibility trial, all experimental diets were run in triplicate.

Before further analysis, feces were pooled per tank ($n$ = 3), freeze-dried, and sieved. Feces samples were homogenized and analyzed for DM, ash, N (CP calculated as N x 6.25), starch, GE, total lipids, fatty acids, and minerals content according to the methodologies described for the algae blend and experimental diets.

## 2.4. Growth trial

From the initial lot, 12 groups of 46 fish (11.3 ± 2.70 g of BW and 10.5 ± 1.04 cm total length) were randomly allocated to 160 L fiberglass tanks within a saltwater recirculation system. The fish were adapted to the new conditions (20.9 ± 0.43°C of water temperature, 35.8 ± 1.29‰ of salinity, 10 L $min^{-1}$ of flow rate, and 12 h of light/dark photoperiod) for 20 days. After acclimation, the experimental diets were randomly assigned to triplicate groups of fish, which were fed by automatic feeders until apparent satiation three times a day, at 9:00, 13:00, and 17:00 h, 7 days a week. The amount of feed supplied to each tank was adjusted according to the presence or absence of feed in the tank (66). Water quality parameters (pH, nitrogenous compounds, and dissolved $O_2$) were monitored daily and maintained within recommended levels for marine fish species (67). The growth trial lasted 12 weeks. Fish were bulk weighed after 5 weeks to monitor weight gain and feed consumption.

Before starting the trial, six fish from the initial lot were sacrificed by anesthetic overdose (1.5 ml $L^{-1}$ of 2-phenoxyethanol, Sigma-Aldrich) and stored at −20°C until whole-body composition analysis. At the end of the growth trial (12 weeks), all fish were fasted for 24 h, anesthetized (60 µl $L^{-1}$ of 2-phenoxyethanol, Sigma-Aldrich), and individually weighted (g) and measured (total length, cm). Six fish per tank were sacrificed by anesthetic overdose (1.5 ml $L^{-1}$ of 2-phenoxyethanol, Sigma-Aldrich) for further whole-body composition analysis, and the remaining fish were sacrificed by a sharp blow to the head. The viscera and liver of 12 fish per tank were weighted (g) to determine the viscerosomatic and hepatosomatic indices. Six fish per tank were collected for skin, muscle, and intestine analysis. Left dorsal skin and muscle were collected and color was immediately assessed. Then, the instrumental texture of the muscle was determined. The right dorsal muscle was collected, snap frozen, and kept at −80°C until nutritional value analysis (DM, CP, total lipids, and fatty acids profile). A section of ∼0.5 cm of the anterior intestine (after the pyloric ceca) was collected, rinsed, and fixed in a 10% neutral-buffered formalin for 24 h, and then transferred to 70% ethanol until histomorphological evaluation.

## 2.5. Intestine histomorphology evaluation

Fixed samples of the anterior intestine from three fish per tank (nine fish per diet) were selected for histological analysis and embedded in paraffin. The embedded tissues were cut into 3 µm sections by a semiautomated rotary microtome (Leica RM 2245, Leica Biosystems, Nussloch, Germany). For quantitative analysis, sections were stained with specific Alcian blue/PAS (pH 2.5) and observed under a light microscope (Olympus BX51; Olympus, Tokyo, Japan) with a camera (Olympus DP50; Olympus). In each section of the samples, cross-sectional perimeter (mm), muscularis externa thickness (µm), submucosa width (µm), lamina propria width (µm), absorption area ($mm^2$), and villus length and width (µm) were measured, and neutral (magenta) and acid (blue) goblet cells were counted using imaging software (Olympus cellSens Dimension Desktop; Olympus). For muscularis externa thickness and submucosa and lamina propria width, eight points of each section were measured, and the average value was calculated. Fold's length was measured in the eight highest folds from the folding tip to the bottom, following the curves of the fold. Goblet cell counts were expressed per villus area.

Intestinal integrity was evaluated using a semi-quantitative analysis: cross-sectional intestinal sections were stained with hematoxylin/eosin and a range of tissue scores set from 1 (normal tissue) to 5 (highly altered) of submucosa and lamina propria cellularity, mucosal folds, inflammatory infiltrates, and enterocytes nucleolus position (68, 69).

## 2.6. Whole-body composition

Before analysis, fish collected for whole-body composition were pooled per tank ($n$ = 12), minced frozen in a commercial meat grinder, and freeze-dried. Whole-fish samples were homogenized and analyzed for DM [ID 934.01, (56)], ash [ID 942.05, (56)], EE [ID 920.39, (56)], and N [ID 990.03, (56)] contents. Crude protein was calculated as N x 6.25, and GE content was determined using an adiabatic bomb calorimeter. All analyses were run in duplicate.

## 2.7. Muscle nutritional value

Dorsal muscle samples were freeze-dried and pooled per two individuals per tank ($n$ = 9). Muscle samples were homogenized

and analyzed for DM and N contents (56), with CP being calculated as N x 6.25. Total lipids of muscle samples were extracted with dichloromethane:methanol (2:1) and determined gravimetrically (60). Fatty acids methyl esters were prepared by direct acid-catalyzed transmethylation (61) and analyzed by gas chromatography as described for the algae blend and experimental diets. The analyses were performed in duplicate.

## 2.8. Skin and muscle color

Skin and muscle color was assessed using a CR-400 Chroma Meter (Konica Minolta Inc., Osaka, Japan) with an aperture of 8 mm, at the CIE D65 standard illuminant. The color was expressed in CIELAB coordinates, where L* measures the degree of lightness (on a scale of 0 to 100, from black to white), a* the degree of redness/greenness (+ red and – green), and b* the degree of yellowness/blueness (+ yellow and – blue). The colorimeter was calibrated against a white plate reference standard (L* = 98.0; a* = 0.3; b* = 2.4; Minolta Co. Ltd.). Color measurements were made by leaning the colorimeter on the surface of the skin and muscle, at three points per fish (54, 66). After flashing, the reflected light values were saved, and the Hue angle ($h^* = \tan^{-1} b^* / a^*$) and Chroma ($C^* = (a^{*2} + b^{*2})^{1/2}$) values were calculated.

## 2.9. Muscle texture

Instrumental dorsal muscle texture was determined using a TA.XT Plus Texture Analyzer equipped with a 2.0-mm diameter probe (Stable Micro Systems, Surrey, UK). The texture profile was obtained in a sequence of two compressions, with a 5-kg load cell, a constant speed of 1.0 mm $s^{-1}$, and a penetration depth of 4.0 mm. Compressions were made 5 s apart at three points of the thickest part of each filet. Texture data were analyzed using Exponent v6 software (Stable Micro Systems), and the texture parameters hardness (N), adhesiveness (J), chewiness (J), springiness, cohesiveness, and resilience were determined (54, 66).

## 2.10. Calculations

Growth performance parameters were calculated based on BW and body length, as follows:

$$Average\ body\ weight\ (ABW) = \frac{final\ BW + initial\ BW}{2}$$

$$Daily\ growth\ index\ (DGI) = \left(\frac{final\ BW^{\frac{1}{3}} - initial\ BW^{\frac{1}{3}}}{days}\right) 100$$

$$Specific\ growth\ rate\ (SGR) = \left(\frac{final\ BW - initial\ BW}{days}\right) 100$$

$$Condition\ factor\ (K) = \left(\frac{final\ BW}{final\ body\ length^3}\right) 100$$

$$Hepatosomatic\ index\ (HSI) = \left(\frac{liver\ weight}{BW}\right) 100$$

$$Viscerosomatic\ index\ (VSI) = \left(\frac{viscera\ weight}{BW}\right) 100$$

Feed efficiency parameters were calculated based on feed intake corrected for the number of fish lost due to mortality and/or sampling, as follows:

$$Voluntary\ feed\ intake\ (VFI) = \frac{dry\ feed\ intake}{ABW\ days}$$

$$Feed\ conversion\ ratio\ (FCR) = \frac{dry\ feed\ intake}{weight\ gain}$$

$$Protein\ efficiency\ ratio\ (PER) = \frac{weight\ gain}{CP\ intake}$$

The apparent digestibility coefficients (ADC) of the experimental diets were calculated based on the amount of yttrium oxide in diets and feces as proposed by Maynard et al. (70):

The nutritional quality indices of lipids in juvenile European seabass filets were calculated according to Chen and Liu (71) as follows:

$$Dry\ matter\ ADC\ (\%) = 100\left(1 - \left(\frac{dietary\ Y_2O_3}{fecal\ Y_2O_3}\right)\right)$$

$$Nutrient\ or\ energy\ ADC\ (\%) = 100\left(1 - \left(\frac{dietary\ Y_2O_3}{fecal\ Y_2O_3}\right)\left(\frac{fecal\ nutrient\ or\ energy}{dietary\ nutrient\ or\ energy}\right)\right)$$

$$Nutrient\ or\ energy\ gain = \frac{final\ carcass\ nutrient\ or\ energy - initial\ carcass\ nutrient\ or\ energy}{ABW\ days}$$

$$Digestible\ nutrient\ or\ energy\ intake = \frac{(dry\ feed\ or\ energy\ intake)\ (nutrient\ or\ energy\ ADC)}{ABW\ days}$$

$$Nutrient\ or\ energy\ retention\ efficiency = \left(\frac{nutrient\ or\ energy\ gain}{digestible\ nutrient\ or\ energy\ intake}\right) 100$$

$$Fecal\ nutrient\ or\ energy\ losses = crude\ nutrient\ or\ energy\ intake\left(1 - \left(\frac{nutrient\ or\ energy\ ADC}{100}\right)\right)$$

$$Nonfecal\ nutrient\ losses = crude\ nutrient\ intake - nutrient\ gain - fecal\ nutrient\ losses$$

$$Nonfecal\ energy\ losses = nonfecal\ nitrogen\ losses\ 25\ kJ\ g^{-1}$$

$$Metabolisable\ energy\ (ME) = digestible\ energy\ intake - nonfecal\ energy\ losses$$

$$Total\ heat\ loss = ME - energy\ gain$$

$$Thrombogenicity\ index\ (TI) = \left(\frac{C14:0 + C16:0 + C18:0}{0.5\ MUFA + 0.5\ PUFA\ n-6 + 3\ PUFA\ n-3 + PUFA\ n-3\ /\ PUFA\ n-6}\right)$$

$$Atherogenicity\ index\ (AI) = \left(\frac{C12:0 + 4\ C14:0 + C16:0}{PUFA\ n-3 + \ PUFA\ n-6 + MUFA}\right)$$

$$Hypocholesterolemic\ to\ hypercholesterolemic\ ratio\ (h/H) = \left(\frac{C18:1\ n-9 + C18:2\ n-6 + C20:4\ n-6 + C18:3\ n-3 + C20:5\ n-3 + C22:6\ n-3}{C14:0 + C16:0}\right)$$

$$Flesh\ lipid\ quality\ (FLQ) = \left(\frac{\text{C20:5 n} - 3 + \text{C22:6 n} - 3}{\text{total fatty acids}}\right) \text{ x } 100$$

## 2.11. Statistical analysis

Data were analyzed using the general linear model (GLM) procedure of SPSS (2009; IBM SPSS statistics V26; IBM, Armonk, NY, USA). The model included the fixed effect of diet (Algae0, Algae2, Algae4, and Algae6) and the random residual error. When statistical differences were observed, multiple comparisons of means were performed using the *post hoc* HSD Tukey test. Effects were considered significant when $p < 0.05$ and a trend when $0.05 \leq p \leq 0.10$.

# 3. Results

## 3.1. Algae blend and experimental diets

The algae blend had moderate CP (347 g kg$^{-1}$ DM) and NDF (126 g kg$^{-1}$ DM) contents, low starch (21.7 g kg$^{-1}$ DM) and GE (16.5 MJ kg$^{-1}$ DM) contents, and high ash content (251 g kg$^{-1}$ DM; Table 1). All amino acids considered essential for European seabass were found in the algae blend (Table 1). Lysine was the most abundant essential amino acid (30.2 g kg$^{-1}$ DM), followed by arginine and leucine (28.7 and 27.3 g kg$^{-1}$ DM, respectively), while methionine and histidine (7.02 and 6.08 g kg$^{-1}$ DM, respectively) were the least abundant. The fatty acids profile of the algae blend was highly unsaturated (Table 1), comprising 37.9% PUFA and 26.8% MUFA. However, the blend had only moderate content of the essential highly unsaturated fatty acid EPA (5.93 g kg$^{-1}$ DM) and no DHA was detected. The algae blend proved to be a good source of macro and trace elements, particularly magnesium, potassium, sodium, aluminum, boron, chromium, copper, iodine, iron, manganese, strontium, and zinc (Supplementary Table S1).

Although experimental diets were formulated to be isoproteic, isolipidic, and isoenergetic, the essential amino acids content was slightly decreased by algae blend inclusion, reflecting its lower content of arginine, histidine, methionine, phenylalanine, and threonine (Table 1). Conversely, the lysine content increased with the inclusion levels of the algae blend. The fatty acids content was globally similar among diets with algae blend inclusion leading to a small decrease in MUFA and an increase in PUFA content (Table 1). The content of magnesium, phosphorus, potassium, sodium, aluminum, cobalt, iron, lithium, manganese, and zinc increased in the diets with algae blend inclusion, while the content of cadmium, chromium, copper, molybdenum, nickel, and selenium decreased (Supplementary Table S1).

## 3.2. Digestibility

The ADC of the nutrients and energy of the experimental diets were greatly affected by algae blend supplementation ($p < 0.05$; Table 2). ADC of DM was 12% higher in Algae4 and Algae6 (72.2 and 73.0%) compared to Algae0 (64.8%); Algae2 did not differ from other diets. Likewise, the algae blend inclusion promoted the ADC of CP and GE, the highest values being found with Algae4 and Algae6 and the lowest with Algae0; Algae2 was similar to other diets. Organic matter and total lipids ADC increased by 9% and 8%, respectively, with algae blend supplementation compared to control (0%), with no differences being observed among inclusion levels.

The ADC of most individual fatty acids was affected by dietary algae blend inclusion ($p < 0.05$; Supplementary Table S2), except C10:0, C17:1 *n*-7, C20:3 *n*-6, and C22:4 *n*-6 ($p > 0.05$). In general, the ADC of individual even-chain fatty acids (ECFA), odd-chain fatty acids (OCFA), branched-chain fatty acids (BCFA), and MUFA were promoted ($p < 0.05$) in diets with algae supplementation, with no differences among inclusion levels. The exceptions were C22:0, which was the highest with Algae6, and C13:0 and C20:1 *n*-11, which were the highest with Algae4 and Algae6 compared to Algae0. Effects on individual PUFA ADC were also marked ($p < 0.05$) but less consistent. ADC of C18:2 *n*-6 and C20:2 *n*-6 were the highest with Algae4 and Algae6 compared to Algae0 and Algae2, and ADC of C18:3 *n*-3 was the lowest and the highest with 2% and 4% algae inclusion, respectively. The ADC of C18:3 *n*-6 was the lowest with Algae0 and the highest with Algae4, while C20:4 *n*-6 was the lowest with Algae6 and the highest with Algae0; Algae2 and Algae4 not differing from the other levels. C18:4 *n*-3, C20:3 *n*-3, C20:4 *n*-3, C21:5 *n*-3, and DHA ADC were the highest with Algae4 and Algae6 compared to the control; Algae2 was similar to other levels. In addition, algae blend dietary inclusion also increased the ADC of total ECFA by 19%, OCFA by 20%, BCFA by 15%, total SFA by 19%, and MUFA by 10% compared to Algae0 ($p < 0.05$), with no differences being observed among inclusion levels. Algae4 and Algae6 improved the ADC of total PUFA *n*-3 by 1% and total PUFA by 2% compared to Algae0, and of PUFA *n*-6 by 2% compared to Algae0 and Algae2 ($p < 0.05$).

The ADC of calcium, potassium, phosphorus, zinc, and selenium were not affected by the inclusion of the algae blend ($p > 0.05$; Table 2). Magnesium ADC was increased by 27% with algae blend supplementation compared to Algae0, with no differences being observed among inclusion levels. The manganese ADC was

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TABLE 2 Apparent digestibility coefficients (%) of nutrients, energy, and minerals of the experimental diets fed to European seabass juveniles.

| | Diet | | | | SEM | *p*-value |
|---|---|---|---|---|---|---|
| | Algae0 | Algae2 | Algae4 | Algae6 | | |
| Dry matter | 64.8$^{a}$ | 68.8$^{ab}$ | 72.2$^{b}$ | 73.0$^{b}$ | 1.13 | 0.003 |
| Organic matter | 74.9$^{a}$ | 78.3$^{b}$ | 80.8$^{b}$ | 81.4$^{b}$ | 0.75 | 0.001 |
| Crude protein | 91.5$^{a}$ | 92.5$^{ab}$ | 93.5$^{b}$ | 93.4$^{b}$ | 0.23 | <0.001 |
| Total lipids | 82.8$^{a}$ | 87.7$^{b}$ | 89.3$^{b}$ | 89.7$^{b}$ | 0.99 | 0.004 |
| Gross energy | 79.8$^{a}$ | 83.2$^{ab}$ | 85.0$^{b}$ | 85.6$^{b}$ | 0.76 | 0.003 |
| **Fatty acids** | | | | | | |
| SFA | 72.7$^{a}$ | 82.8$^{b}$ | 88.2$^{b}$ | 88.4$^{b}$ | 1.91 | 0.001 |
| MUFA | 85.0$^{a}$ | 91.3$^{b}$ | 94.3$^{b}$ | 94.3$^{b}$ | 1.17 | 0.001 |
| PUFA | 94.6$^{a}$ | 95.2$^{ab}$ | 96.5$^{b}$ | 96.4$^{b}$ | 0.30 | 0.006 |
| PUFA *n*-3 | 95.6$^{a}$ | 96.2$^{ab}$ | 97.1$^{b}$ | 97.0$^{b}$ | 0.31 | 0.025 |
| PUFA *n*-6 | 91.0$^{a}$ | 91.1$^{a}$ | 93.7$^{b}$ | 93.5$^{b}$ | 0.37 | <0.001 |
| **Elements** | | | | | | |
| Calcium | 86.3 | 85.9 | 86.8 | 87.2 | 0.38 | 0.186 |
| Magnesium | 59.7$^{a}$ | 72.0$^{b}$ | 75.9$^{b}$ | 79.1$^{b}$ | 1.91 | <0.001 |
| Potassium | 95.8 | 96.5 | 96.5 | 96.8 | 0.30 | 0.236 |
| Phosphorus | 64.0 | 64.1 | 61.8 | 61.9 | 1.22 | 0.418 |
| Manganese | 71.6$^{ab}$ | 69.6$^{ab}$ | 67.0$^{a}$ | 72.7$^{b}$ | 1.10 | 0.029 |
| Iron | 14.2$^{c}$ | 12.1$^{bc}$ | 10.8$^{b}$ | 4.00$^{a}$ | 0.556 | <0.001 |
| Copper | 81.2$^{b}$ | 76.4$^{a}$ | 78.3$^{ab}$ | 77.2$^{a}$ | 0.55 | 0.006 |
| Selenium | 14.7 | 15.6 | 14.1 | 15.9 | 1.36 | 0.785 |

Algae0, commercial-based diet without algae blend inclusion (control diet); Algae2, control diet with 2% algae blend inclusion; Algae4, control diet with 4% algae blend inclusion; Algae6, control diet with 6% algae blend inclusion; SEM, standard error of the mean; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.
$^{a-c}$ Means in the same line with different superscripts are statistically different ($p < 0.05$).

the lowest with Algae4 and the highest with Algae6; Algae0 and Algae2 not differing from the other diets. Copper ADC was reduced with algae supplementation; the lowest values were found with Algae2 and Algae6 and the highest with Algae0; Algae4 not differing from the other diets. Similarly, iron ADC decreased with increasing levels of the algae blend, with Algae6 being 72% lower than Algae0 (Table 2).

The effects of algae blend inclusion on N, lipid, and energy balances of European seabass juveniles are shown in Table 3. Digestible N, lipid, energy intake, and lipid gain gradually increased with algae blend inclusion ($p < 0.05$). Nitrogen and energy gains were promoted by the algae blend ($p < 0.05$), but no differences were observed among inclusion levels. Nitrogen retention efficiency was not affected by algae supplementation, while lipid and energy retention efficiencies were increased ($p < 0.05$), regardless of the inclusion level. Fecal N losses were the lowest with Algae4 and the highest with Algae0, while non-fecal N losses increased with the algae blend inclusion levels ($p < 0.05$). Fecal and non-fecal lipid losses were promoted by algae blend supplementation ($p < 0.05$), with no differences being observed among inclusion levels. Non-fecal energy losses were the highest with Algae6 compared to Algae0 ($p < 0.05$). Metabolizable energy gradually increased with algae blend supplementation levels ($p < 0.05$), with Algae6 being 30.7% higher than Algae0. Total heat loss was not affected by the dietary algae blend ($p > 0.05$).

## 3.3. Growth performance and feed utilization

Most growth performance and feed utilization parameters were affected by algae blend feeding ($p < 0.05$; Table 4). Final fish BW and length were improved, with the BW of fish fed Algae6 being 70% higher than that of fish fed Algae0. Algae blend supplementation promoted DGI, VSI, and HIS compared to the control (0%), but no differences were observed among inclusion levels. The lowest FCR was observed with 2% and 4% of algae blend and the highest with Algae0. The PER was the highest with Algae4, not differing from Algae2 and Algae 6. The algae blend also improved the condition factor, which was highest at the 4% and 6% inclusion levels.

Feed intake of DM and CP was promoted in diets with algae supplementation, with no differences among inclusion levels. Feed intake of EE and GE was the highest at 6% and 4% algae inclusion, the latter not differing from 2% (Table 4).

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TABLE 3 Nutrient and energy balances of European seabass juveniles fed the experimental diets.

| | Diet | | | | SEM | *p*-value |
|---|---|---|---|---|---|---|
| | Algae0 | Algae2 | Algae4 | Algae6 | | |
| **Nitrogen (N) balance (mg 100 g ABW$^{-1}$ day$^{-1}$)** | | | | | | |
| Digestible N (DN) intake | 115$^{a}$ | 130$^{b}$ | 132$^{bc}$ | 142$^{c}$ | 2.7 | <0.001 |
| N gain | 33.6$^{a}$ | 40.0$^{b}$ | 41.8$^{b}$ | 43.2$^{b}$ | 1.20 | <0.001 |
| N retention efficiency (% DN) | 29.3 | 30.7 | 31.7 | 30.4 | 0.65 | 0.147 |
| Fecal N losses | 10.6$^{b}$ | 10.5$^{ab}$ | 9.13$^{a}$ | 10.1$^{ab}$ | 0.30 | 0.036 |
| Non-fecal N losses | 81.0$^{a}$ | 90.4$^{bc}$ | 89.8$^{ab}$ | 99.2$^{c}$ | 2.00 | 0.002 |
| **Lipid (L) balance (mg 100 g ABW$^{-1}$ day$^{-1}$)** | | | | | | |
| Digestible L (DL) intake | 197$^{a}$ | 231$^{b}$ | 239$^{bc}$ | 260$^{c}$ | 4.7 | <0.001 |
| L gain | 104$^{a}$ | 211$^{b}$ | 232$^{bc}$ | 255$^{c}$ | 7.6 | <0.001 |
| L retention efficiency (% DL) | 53.1$^{a}$ | 91.3$^{b}$ | 94.9$^{b}$ | 96.0$^{b}$ | 3.25 | <0.001 |
| Fecal L losses | 33.6$^{b}$ | 20.5$^{a}$ | 16.3$^{a}$ | 16.9$^{a}$ | 2.20 | 0.002 |
| Non-fecal L losses | 92.4$^{b}$ | 20.2$^{a}$ | 12.1$^{a}$ | 10.6$^{a}$ | 6.90 | <0.001 |
| **Energy (E) balance (kJ kg ABW$^{-1}$ day$^{-1}$)** | | | | | | |
| Digestible E (DE) intake | 259$^{a}$ | 298$^{b}$ | 308$^{bc}$ | 336$^{c}$ | 7.2 | <0.001 |
| Metabolizable E | 238$^{a}$ | 276$^{b}$ | 286$^{bc}$ | 311$^{c}$ | 6.8 | <0.001 |
| E gain | 84.5$^{a}$ | 129$^{b}$ | 136$^{b}$ | 143$^{b}$ | 4.0 | <0.001 |
| E retention efficiency (% DE) | 32.7$^{a}$ | 43.3$^{b}$ | 44.1$^{b}$ | 42.7$^{b}$ | 1.26 | <0.001 |
| Fecal E losses | 65.4 | 60.3 | 54.5 | 56.4 | 2.55 | 0.067 |
| Non-fecal E losses | 20.2$^{a}$ | 22.5$^{bc}$ | 22.4$^{ab}$ | 24.7$^{c}$ | 0.50 | 0.002 |
| Total heat loss | 154 | 147 | 150 | 168 | 5.7 | 0.118 |

Algae0, commercial-based diet without algae blend inclusion (control diet); Algae2, control diet with 2% algae blend inclusion; Algae4, control diet with 4% algae blend inclusion; Algae6, control diet with 6% algae blend inclusion; SEM, standard error of the mean; ABW, average body weight.

$^{a-c}$ Means in the same line with different superscripts are statistically different ($p < 0.05$).

The survival rate was not affected by algae blend supplementation ($p > 0.05$; Table 4).

## 3.4. Histomorphology of anterior intestine

The overall integrity of the anterior intestine was well preserved in all fish, but algae blend supplementation had a significant impact on its morphology ($p < 0.05$; Figure 1, Table 5).

Most quantitative parameters were affected by the dietary inclusion of algae ($p < 0.05$), except for muscularis thickness, number of acid goblet cells, and number of goblet cells per villus area ($p > 0.05$). The cross-sectional perimeter, the submucosa width, and the villus length increased with algae inclusion levels, being the highest in fish fed Algae4 and Algae6 and the lowest in fish fed Algae0 and Algae2 for submucosa width; Algae2 was similar to other inclusion levels for cross-sectional perimeter and villus length. Compared to Algae0, lamina propria width was the highest in fish fed Algae4 while the highest absorption area was observed in fish fed Algae6. Goblet cell counts were the highest in fish fed Algae2 and Algae6.

Submucosal and lamina propria cellularity, mucosal folds, inflammatory infiltrates, and enterocyte nucleolus position ranged from normal to slightly altered tissue, not being significantly affected by algae blend inclusion ($p > 0.05$).

## 3.5. Whole-body composition

After 12 weeks of feeding, whole-body moisture and EE gradually decreased and increased, respectively, with the inclusion levels of the algae blend ($p < 0.05$; Table 6). Whole-body GE content of fish fed algae-supplemented diets was enhanced; no differences were observed among inclusion levels ($p < 0.05$). Ash and CP contents were not affected ($p > 0.05$).

## 3.6. Muscle composition

Muscle moisture content decreased whereas total lipids and fatty acids content increased with algae supplementation compared to control ($p < 0.05$; Table 7), with no differences observed among levels. Algae supplementation did not affect muscle CP content ($p > 0.05$).

The muscle fatty acids profile was greatly affected by algae blend supplementation ($p < 0.05$; Table 7). Total SFA remained

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TABLE 4 Growth performance and feed utilization of European seabass juveniles fed the experimental diets.

| | Diet | | | | SEM | *p*-value |
|---|---|---|---|---|---|---|
| | Algae0 | Algae2 | Algae4 | Algae6 | | |
| **Growth** | | | | | | |
| Final body weight (g) | 37.3[a] | 54.4[b] | 56.6[b] | 62.9[c] | 1.35 | <0.001 |
| Final length (cm) | 15.6[a] | 17.1[b] | 17.1[b] | 17.7[c] | 0.14 | <0.001 |
| Specific growth rate | 1.23[a] | 1.62[b] | 1.65[b] | 1.77[b] | 0.009 | <0.001 |
| Feed conversion ratio | 1.35[b] | 1.24[a] | 1.22[a] | 1.26[ab] | 0.025 | 0.021 |
| Protein efficiency ratio | 1.41[a] | 1.53[ab] | 1.56[b] | 1.50[ab] | 0.028 | 0.024 |
| Condition factor | 0.96[a] | 1.07[b] | 1.11[c] | 1.12[c] | 0.009 | <0.001 |
| Daily growth index | 1.13[a] | 1.59[b] | 1.64[b] | 1.79[b] | 0.060 | <0.001 |
| **Somatic indices (%)** | | | | | | |
| Viscerosomatic index | 6.14[a] | 7.53[b] | 7.88[b] | 8.38[b] | 0.245 | <0.001 |
| Hepatosomatic index | 0.953[a] | 1.31[b] | 1.33[b] | 1.42[b] | 0.036 | <0.001 |
| **Voluntary feed intake (g kg ABW$^{-1}$ day$^{-1}$)** | | | | | | |
| Dry matter | 14.9[a] | 16.7[b] | 16.8[b] | 18.0[b] | 0.32 | 0.001 |
| Crude protein | 7.82[a] | 8.81[b] | 8.80[b] | 9.53[b] | 0.170 | 0.001 |
| Ether extract | 2.30[a] | 2.52[ab] | 2.56[bc] | 2.77[c] | 0.049 | 0.001 |
| Gross energy (kJ kg ABW$^{-1}$ day$^{-1}$) | 324[a] | 359[b] | 363[bc] | 392[c] | 7.0 | 0.001 |
| Survival (%) | 100 | 99.3 | 98.6 | 100 | 0.52 | 0.219 |

Algae0, commercial-based diet without algae blend inclusion (control diet); Algae2, control diet with 2% algae blend inclusion; Algae4, control diet with 4% algae blend inclusion; Algae6, control diet with 6% algae blend inclusion; SEM, standard error of the mean; ABW, average body weight.
[a–c] Means in the same line with different superscripts are statistically different ($p < 0.05$).

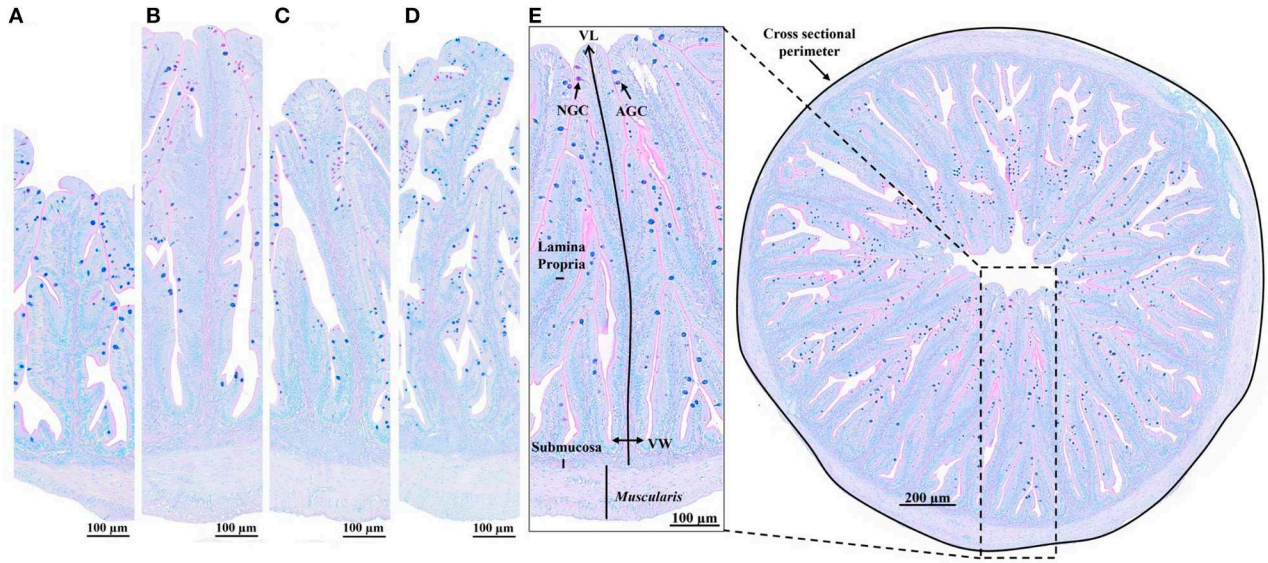


FIGURE 1
Histological sections (Alcian blue/PAS staining, pH = 2.5) of the anterior intestine of European seabass fed **(A)** commercial-based diet without algae blend inclusion (control diet; Algae0), **(B)** control diet with 2% algae blend inclusion (Algae2), **(C)** control diet with 4% algae blend inclusion (Algae4), and **(D)** control diet with 6% algae blend inclusion (Algae6). **(E)** Measured parameters: cross-sectional perimeter, villus length (VL), villus width (VW), muscularis, submucosa and lamina propria width, acid goblet cells (AGC, blue), and neutral goblet cells (NGC, pink).

unchanged, reflecting the effect on C16:0 ($p > 0.05$). Total MUFA, C16:1 *n*-7, and C18:1 *n*-9 proportions were the highest in fish fed with 6% algae blend and the lowest in those fed control (0%) diets. Conversely, C20:1 *n*-9 and C20:1 *n*-11 decreased with algae blend inclusion compared to Algae0. Dietary inclusion of the algae blend decreased the proportions of all individual *n*-6 PUFA, leading

TABLE 5 Anterior intestine morphology of European seabass juveniles fed the experimental diets.

| | Diet | | | | SEM | *p*-value |
|---|---|---|---|---|---|---|
| | Algae0 | Algae2 | Algae4 | Algae6 | | |
| **Quantitative analysis** | | | | | | |
| Cross-sectional perimeter (mm) | 7.70$^{a}$ | 8.87$^{ab}$ | 8.97$^{b}$ | 9.77$^{b}$ | 0.311 | <0.001 |
| Muscularis thickness (μm) | 74.6 | 92.9 | 98.6 | 96.7 | 7.76 | 0.131 |
| Submucosa width (μm) | 21.9$^{a}$ | 25.6$^{a}$ | 32.3$^{b}$ | 30.9$^{b}$ | 1.37 | <0.001 |
| Lamina propria width (μm) | 17.4$^{a}$ | 21.3$^{ab}$ | 22.5$^{b}$ | 20.0$^{ab}$ | 1.28 | 0.046 |
| Absorption area ($mm^2$) | 2.16$^{a}$ | 2.69$^{ab}$ | 2.79$^{ab}$ | 3.13$^{b}$ | 0.228 | 0.038 |
| Villus length (μm) | 774$^{a}$ | 927$^{ab}$ | 966$^{b}$ | 1033$^{b}$ | 44.7 | 0.002 |
| Villus width (μm) | 104$^{a}$ | 124$^{b}$ | 132$^{b}$ | 130$^{b}$ | 5.2 | 0.002 |
| Goblet cells number | 42.9$^{a}$ | 59.5$^{b}$ | 56.4$^{ab}$ | 62.2$^{b}$ | 3.98 | 0.009 |
| Neutral goblet cells | 4.46 | 10.2 | 9.97 | 10.8 | 1.891 | 0.082 |
| Acid goblet cells | 38.5 | 49.3 | 46.4 | 51.4 | 4.48 | 0.209 |
| Goblet cells per villus area (n° $mm^{2-1}$) | 20.4 | 23.6 | 20.6 | 20.3 | 1.74 | 0.487 |
| Neutral goblet cells | 2.07 | 4.37 | 3.62 | 3.89 | 0.928 | 0.343 |
| Acid goblet cells | 18.3 | 19.2 | 17.0 | 16.4 | 1.65 | 0.616 |
| **Semi-quantitative analysis** | | | | | | |
| Submucosa cellularity | 1.28 | 1.44 | 1.33 | 1.56 | 0.172 | 0.675 |
| Lamina propria cellularity | 2.44 | 2.39 | 2.50 | 2.56 | 0.115 | 0.763 |
| Mucosal folds | 1.67 | 1.78 | 1.44 | 1.61 | 0.214 | 0.739 |
| Inflammatory infiltrates | 2.50 | 2.50 | 2.50 | 2.67 | 0.125 | 0.723 |
| Enterocytes nucleolus position | 2.11 | 1.83 | 1.94 | 1.78 | 0.178 | 0.571 |

Algae0, commercial-based diet without algae blend inclusion (control diet); Algae2, control diet with 2% algae blend inclusion; Algae4, control diet with 4% algae blend inclusion; Algae6, control diet with 6% algae blend inclusion; SEM, standard error of the mean.
[a,b]Means in the same line with different superscripts are statistically different ($p < 0.05$).

TABLE 6 Whole-body composition (g 100 $g^{-1}$, wet weight) of European seabass juveniles fed the experimental diets.

| | Diet | | | | SEM | *p*-value |
|---|---|---|---|---|---|---|
| | Algae0 | Algae2 | Algae4 | Algae6 | | |
| Moisture | 69.0$^{c}$ | 64.0$^{b}$ | 62.8$^{ab}$ | 62.3$^{a}$ | 0.29 | <0.001 |
| Ash | 4.67 | 4.35 | 4.39 | 4.13 | 0.225 | 0.457 |
| Crude protein | 18.3 | 18.1 | 18.6 | 18.4 | 0.14 | 0.239 |
| Ether extract | 8.89$^{a}$ | 14.0$^{b}$ | 15.0$^{bc}$ | 15.9$^{c}$ | 0.335 | <0.001 |
| Gross energy (kJ $g^{-1}$) | 7.42$^{a}$ | 9.01$^{b}$ | 9.30$^{b}$ | 9.42$^{b}$ | 0.143 | <0.001 |

Algae0, commercial-based diet without algae blend inclusion (control diet); Algae2, control diet with 2% algae blend inclusion; Algae4, control diet with 4% algae blend inclusion; Algae6, control diet with 6% algae blend inclusion; SEM, standard error of the mean.
[a–c] Means in the same line with different superscripts are statistically different ($p < 0.05$).
Initial whole-body composition (g 100 $g^{-1}$, wet weight): moisture: 70.5; ash: 4.85; crude protein: 16.6; ether extract: 7.54; gross energy: 6.81 (kJ $g^{-1}$).

to a decrease in total *n*-6 PUFA by 24.5%. Effects on *n*-3 PUFA differed among individual fatty acids. Dietary inclusion of the algae blend increased C18:4 *n*-3 and C20:4 *n*-3 and decreased C21:5 *n*-3, C22:5 *n*-3, and DHA proportions in muscle. Muscle EPA remained unchanged ($p > 0.05$). Overall, the total *n*-3 PUFA proportion gradually decreased with algae supplementation, with Algae6 being 7.8% lower than Algae0. The observed decrease in PUFA *n*-6 and *n*-3 led to a decrease in total PUFA and *n*-6/*n*-3 ratio with algae blend inclusion levels.

Although the PUFA proportions decreased with the algae blend inclusion, the higher lipid and fatty acids content of algae blend-fed European seabass muscle led to an increase in the essential fatty acids (EPA + DHA) content by nearly 43%; no differences were found among inclusion levels.

Algae blend supplementation increased the thrombogenicity and the atherogenicity indices ($p < 0.05$; Table 7); no differences were observed among levels in the former, while a gradual increase with increasing algae inclusion levels was observed in the latter.

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TABLE 7 Muscle nutritional value (g 100 $g^{-1}$ wet weight, ww), fatty acids profile (g 100 $g^{-1}$ total fatty acids), and lipid quality indices of European seabass juveniles fed the experimental diets.

| | Diet | | | | SEM | *p*-value |
|---|---|---|---|---|---|---|
| | Algae0 | Algae2 | Algae4 | Algae6 | | |
| **Nutritional value** | | | | | | |
| Moisture | 75.6[b] | 74.1[a] | 73.5[a] | 72.9[a] | 0.30 | <0.001 |
| Crude protein | 19.4 | 19.5 | 19.5 | 19.5 | 0.16 | 0.918 |
| Total lipids | 2.34[a] | 3.32[b] | 3.62[b] | 4.08[b] | 0.227 | <0.001 |
| Total fatty acids | 2.26[a] | 3.22[b] | 3.26[b] | 3.78[b] | 0.169 | <0.001 |
| **Fatty acids** | | | | | | |
| **Saturated fatty acids** | | | | | | |
| Total SFA[1,2,3] | 32.4 | 32.6 | 32.8 | 32.9 | 0.21 | 0.442 |
| **Even-chain fatty acids** | | | | | | |
| C14:0 | 3.64[a] | 4.12[b] | 4.49[c] | 4.72[d] | 0.052 | <0.001 |
| C16:0 | 21.7 | 21.7 | 21.7 | 21.7 | 0.12 | 0.990 |
| C18:0 | 5.08[c] | 4.81[bc] | 4.53[ab] | 4.30[a] | 0.108 | <0.001 |
| C20:0 | 0.177[a] | 0.187[b] | 0.200[c] | 0.213[d] | 0.0020 | <0.001 |
| Sum[1] | 30.9 | 31.1 | 31.2 | 31.2 | 0.20 | 0.751 |
| **Odd-chain fatty acids** | | | | | | |
| C15:0 | 0.402 | 0.416 | 0.426 | 0.438 | 0.0090 | 0.062 |
| C17:0 | 0.442 | 0.434 | 0.441 | 0.465 | 0.0140 | 0.437 |
| Sum[2] | 0.86 | 0.869 | 0.886 | 0.924 | 0.0230 | 0.221 |
| **Branched-chain fatty acids** | | | | | | |
| *iso*-C17:0 | 0.302[a] | 0.318[ab] | 0.339[bc] | 0.357[c] | 0.0090 | 0.001 |
| Sum[3] | 0.620[a] | 0.666[ab] | 0.705[bc] | 0.746[c] | 0.0180 | <0.001 |
| **Monounsaturated fatty acids** | | | | | | |
| C16:1 *n*-7 | 6.09[a] | 6.75[b] | 7.26[c] | 7.71[d] | 0.089 | <0.001 |
| C16:1 *n*-9 | 0.577 | 0.565 | 0.560 | 0.550 | 0.0130 | 0.552 |
| C18:1 *n*-7 | 3.24 | 3.26 | 3.30 | 3.42 | 0.074 | 0.356 |
| C18:1 *n*-9 | 20.3[a] | 20.8[ab] | 21.2[ab] | 21.8[b] | 0.34 | 0.027 |
| C20:1 *n*-9 | 1.97[c] | 1.91[bc] | 1.84[ab] | 1.77[a] | 0.022 | <0.001 |
| C20:1 *n*-11 | 0.336[b] | 0.319[ab] | 0.311[a] | 0.306[a] | 0.0060 | 0.006 |
| C22:1 *n*-9 | 0.255 | 0.249 | 0.248 | 0.248 | 0.0080 | 0.892 |
| C22:1 *n*-11 | 1.48 | 1.43 | 1.38 | 1.33 | 0.042 | 0.076 |
| C24:1 *n*-9 | 0.487 | 0.441 | 0.450 | 0.460 | 0.0170 | 0.271 |
| Total MUFA[4] | 34.9[a] | 36.0[ab] | 36.8[bc] | 37.8[c] | 0.41 | <0.001 |
| **Polyunsaturated fatty acids** | | | | | | |
| C16:2 *n*-4 | 0.348[a] | 0.408[b] | 0.421[b] | 0.448[c] | 0.0060 | <0.001 |
| C16:3 *n*-4 | 0.260[a] | 0.296[b] | 0.323[bc] | 0.355[c] | 0.0090 | <0.001 |
| C16:4 *n*-1 | 0.485[a] | 0.515[b] | 0.530[b] | 0.555[c] | 0.0060 | <0.001 |
| C18:2 *n*-6 | 5.36[d] | 4.81[c] | 4.41[b] | 4.04[a] | 0.057 | <0.001 |
| C18:3 *n*-3 | 0.797[c] | 0.777[b] | 0.767[ab] | 0.757[a] | 0.0050 | <0.001 |
| C18:3 *n*-6 | 0.211[c] | 0.184[b] | 0.150[a] | 0.136[a] | 0.0040 | <0.001 |
| C18:4 *n*-3 | 1.06[a] | 1.12[a] | 1.21[b] | 1.30[c] | 0.020 | <0.001 |

*(Continued)*

TABLE 7 (Continued)

| | Diet | | | | SEM | *p*-value |
|---|---|---|---|---|---|---|
| | Algae0 | Algae2 | Algae4 | Algae6 | | |
| C20:2 *n*-6 | 0.376$^{c}$ | 0.349$^{b}$ | 0.321$^{a}$ | 0.318$^{a}$ | 0.0060 | <0.001 |
| C20:4 *n*-3 | 0.410$^{a}$ | 0.416$^{ab}$ | 0.433$^{bc}$ | 0.445$^{c}$ | 0.0050 | <0.001 |
| C20:4 *n*-6 | 0.941$^{b}$ | 0.807$^{a}$ | 0.758$^{a}$ | 0.684$^{a}$ | 0.0320 | <0.001 |
| C20:5 *n*-3 (EPA) | 9.09 | 9.05 | 9.22 | 9.33 | 0.136 | 0.433 |
| C21:5 *n*-3 | 0.300$^{c}$ | 0.267$^{bc}$ | 0.236$^{ab}$ | 0.216$^{a}$ | 0.0110 | <0.001 |
| C22:5 *n*-3 | 1.26$^{c}$ | 1.23$^{bc}$ | 1.15$^{ab}$ | 1.07$^{a}$ | 0.028 | <0.001 |
| C22:6 *n*-3 (DHA) | 11.43$^{d}$ | 10.8$^{c}$ | 10.1$^{b}$ | 9.31$^{a}$ | 0.121 | <0.001 |
| Sum PUFA *n*-3[5] | 24.4$^{c}$ | 23.7$^{bc}$ | 23.2$^{ab}$ | 22.5$^{a}$ | 0.25 | <0.001 |
| Sum PUFA *n*-6[6] | 7.11$^{d}$ | 6.36$^{c}$ | 5.84$^{b}$ | 5.37$^{a}$ | 0.077 | <0.001 |
| Total PUFA[5,6,7] | 32.7$^{c}$ | 31.4$^{b}$ | 30.4$^{ab}$ | 29.3$^{a}$ | 0.31 | <0.001 |
| **Ratios** | | | | | | |
| PUFA/SFA | 1.01$^{c}$ | 0.961$^{b}$ | 0.928$^{ab}$ | 0.891$^{a}$ | 0.0100 | <0.001 |
| *n*-6/*n*-3 | 0.291$^{d}$ | 0.268$^{c}$ | 0.251$^{b}$ | 0.239$^{a}$ | 0.0030 | <0.001 |
| EPA+DHA (mg 100 g$^{-1}$ ww) | 441$^{a}$ | 611$^{b}$ | 602$^{b}$ | 674$^{b}$ | 0318 | <0.001 |
| **Lipid quality indices** | | | | | | |
| AI | 0.577$^{a}$ | 0.611$^{b}$ | 0.636$^{c}$ | 0.652$^{d}$ | 0.0040 | <0.001 |
| TI | 0.295$^{a}$ | 0.314$^{b}$ | 0.316$^{b}$ | 0.325$^{b}$ | 0.0030 | <0.001 |
| h/H ratio | 1.94$^{c}$ | 1.87$^{b}$ | 1.82$^{ab}$ | 1.78$^{a}$ | 0.015 | <0.001 |
| Flesh quality score | 20.5$^{c}$ | 19.8$^{bc}$ | 19.4$^{ab}$ | 18.6$^{a}$ | 0.22 | <0.001 |

Algae0, commercial-based diet without algae blend inclusion (control diet); Algae2, control diet with 2% algae blend inclusion; Algae4, control diet with 4% algae blend inclusion; Algae6, control diet with 6% algae blend inclusion; SEM, standard error of the mean; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; *n*-6/*n*-3 ratio, *n*-6 PUFA to sum of *n*-3 PUFA ratio; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; AI, atherogenicity index; TI, thrombogenicity index; h/H ratio, hypocholesterolemic to hypercholesterolemic fatty acids ratio.

[1]Sum includes C10:0, C12:0, C22:0, and C24:0.

[2]Sum includes C13:0.

[3]Sum includes *iso*-C14:0, *iso*-C15:0, *anteiso*-C15:0, *iso*-C16:0, and *anteiso*-C17:0.

[4]Sum includes C14:1 *n*-5, C17:1 *n*-7, and C20:1 *n*-7.

[5]Sum includes 20:3 *n*-3.

[6]Sum includes C20:3 *n*-6, C22:2 *n*-6, and C22:4 *n*-6.

[7]Sum includes 18:3 *n*-4.

[a–d]Means in the same line with different superscripts are statistically different ($p < 0.05$).

The h/H ratio and flesh quality score decreased with algae inclusion levels ($p < 0.05$), in fish fed Algae0 being the highest and in those fed Algae6 being the lowest.

## 3.7. Color of skin and muscle, and texture of muscle

Dorsal skin color parameters were affected by algae blend feeding ($p < 0.05$; Table 8). Skin brightness (L*) was the highest in control fish and the lowest in those fed Algae2, with Algae4 and Algae6 not differing from Algae0 and Algae2. The algae blend reduced the greenness (a*) of the skin compared to the control, while Alage4 and Algae6 increased the yellowness (b*) compared to Algae2, although not differing from Algae0. Chroma (C*) was the highest in the skin of fish fed Algae4 and the lowest in those fed Algae2. The skin hue angle (h) of fish fed Algae4 and Algae6 was lower compared to those fed Algae0 and Algae2, reflecting a less greenish skin tone.

Muscle brightness (L*) and greenness (a*) were not affected by algae blend inclusion levels, while yellowness (b*) was the highest in fish fed Algae0 and the lowest in those fed Algae2 and Algae4 (Table 7); Algae6 fish did not differ from other diets. Compared to the control, algae blend inclusion reduced muscle chroma (C*), with no differences among inclusion levels. Conversely, the hue angle (h) was increased by algae feeding, with the highest value found in the muscle of fish fed Algae4 (more greenish) and the lowest in that of fish fed Algae0 (Table 8).

Regarding texture (Table 8), muscle hardness was the lowest in fish fed Algae4 and Algae6 and the highest in those fed the control diet (Algae0); Algae2 did not differ from the other levels. Muscle adhesiveness increased in fish fed algae blend, regardless of supplementation level. Muscle springiness, cohesiveness, and chewiness were not affected by algae supplementation. A trend

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TABLE 8 Skin and muscle color and muscle texture of European seabass juveniles fed the experimental diets.

| | Diet | | | | SEM | *p*-value |
|---|---|---|---|---|---|---|
| | Algae0 | Algae2 | Algae4 | Algae6 | | |
| **Color** | | | | | | |
| **Skin** | | | | | | |
| L* | 69.1$^{b}$ | 63.4$^{a}$ | 64.7$^{ab}$ | 67.3$^{ab}$ | 1.40 | 0.027 |
| a* | −5.76$^{a}$ | −4.66$^{b}$ | −4.59$^{b}$ | −4.55$^{b}$ | 0.144 | <0.001 |
| b* | 9.67$^{ab}$ | 9.07$^{a}$ | 10.6$^{b}$ | 10.4$^{b}$ | 0.327 | 0.007 |
| C* | 11.3$^{ab}$ | 10.2$^{a}$ | 11.5$^{b}$ | 11.3$^{ab}$ | 0.31 | 0.020 |
| h | 121$^{b}$ | 118$^{b}$ | 114$^{a}$ | 114$^{a}$ | 0.9 | <0.001 |
| **Muscle** | | | | | | |
| L* | 51.4 | 52.5 | 51.7 | 50.9 | 0.92 | 0.637 |
| a* | −3.26 | −3.60 | −3.57 | −3.29 | 0.161 | 0.308 |
| b* | 6.65$^{b}$ | 5.54$^{a}$ | 5.25$^{a}$ | 5.70$^{ab}$ | 0.260 | 0.002 |
| C* | 7.51$^{b}$ | 6.72$^{a}$ | 6.43$^{a}$ | 6.67$^{a}$ | 0.203 | 0.002 |
| h | 118$^{a}$ | 123$^{ab}$ | 125$^{b}$ | 120$^{ab}$ | 1.9 | 0.038 |
| **Muscle texture** | | | | | | |
| Hardness | 1.46$^{b}$ | 1.26$^{ab}$ | 1.20$^{a}$ | 1.06$^{a}$ | 0.068 | 0.001 |
| Adhesiveness | −0.111$^{a}$ | −0.0542$^{b}$ | −0.0645$^{b}$ | −0.0560$^{b}$ | 0.00886 | <0.001 |
| Springiness | 1.17 | 1.19 | 1.23 | 1.32 | 0.057 | 0.264 |
| Cohesiveness | 0.242 | 0.265 | 0.269 | 0.255 | 0.0148 | 0.573 |
| Chewiness | 0.426 | 0.377 | 0.403 | 0.336 | 0.0403 | 0.437 |
| Resilience | 0.596 | 0.738 | 0.654 | 0.904 | 0.0885 | 0.087 |

Algae0, commercial-based diet without algae blend inclusion (control diet); Algae2, control diet with 2% algae blend inclusion; Algae4, control diet with 4% algae blend inclusion; Algae6, control diet with 6% algae blend inclusion; SEM, standard error of the mean; L*, lightness degree; a*, redness/greeness degree; b*, yellowness/blueness degree; C*, chroma value; h, hue angle value.

[a,b]Means in the same line with different superscripts are statistically different ($p < 0.05$).

toward increased muscle resilience with algae supplementation was observed.

## 4. Discussion

To ensure aquaculture sustainability, challenges related to aquafeed formulation and ingredient selection must be addressed, in line with the circular economy, the Blue Growth strategy of the European Union, and the Sustainable Development Goals of the 2030 Agenda. In recent years, algae have emerged as alternative aquafeed ingredients due to their nutritional and functional values and lower environmental footprint (13, 72), particularly macroalgae produced in integrated multi-trophic aquaculture (IMTA) systems and microalgae produced locally. Although dietary supplementation of micro- or macroalgae species as sustainable alternative aquafeeds to fishmeal and fish oil (13, 73, 74) or plant source ingredients (34, 75) has been assessed, the synergetic effects of the blend of micro- and macroalgae remain largely unexploited. The present study addressed this gap and unveiled the potential of dietary supplementation of a commercial blend of macro- (*Ulva* sp. and *G. gracilis*) and microalgae (*C. vulgaris* and *N. oceanica*) species up to 6% (DM basis) in digestibility, growth performance, and muscle nutritional value and quality of European seabass juveniles.

The functional potential of this algae blend has recently been suggested based on its chemical composition and bacteriostatic and bactericidal activities evidenced *in vitro* against some of the most common fish pathogenic bacteria (23). To the best of our knowledge, no other study has yet evaluated an algae blend composed of these four species in an *in vivo* study. The composition of macro- and microalgae is known to vary between species and within species with biotic and abiotic growth factors (76). Nonetheless, the overall proximate composition and amino acids content of the blend previously analyzed (23) are in broad agreement with the present results. The most relevant differences were in the polysaccharide content and the fatty acids content and profile. The commercial blend used in the present study had lower polysaccharides content (148 vs. 341 g $kg^{-1}$) and total FA content (54.9 vs. 79.0 g $kg^{-1}$), but higher PUFA *n*-3 (22.0 vs. 17.6% total FA) and EPA (10.8 vs. 6.91% total FA) proportions than that previously reported (23). The high content of macro (e.g., magnesium, potassium, and sodium) and trace elements (e.g., iron, manganese, and zinc) reported here further support

the interest in the algae blend as functional aquafeed ingredients. However, the levels of toxic elements such as aluminum, arsenic, and copper may limit the inclusion level of the algae blend in fish diets.

In the present study, the commercial blend of *Ulva* sp., *G. gracilis*, *C. vulgaris,* and *N. oceanica* replaced protein-rich plant ingredients and had a positive effect if included up to 6% (DM basis): growth performance, feed intake, utilization efficiency, and body composition of *D. labrax* juveniles were enhanced compared to those fed the control diet (Algae0), a commercial-type formulated plant-based diet with moderate (125 g $kg^{-1}$ DM basis) inclusion of fishmeal. These remarkable results were mostly unexpected as most studies evaluating dietary inclusion of mixtures of macroalgae species, microalgae species, or a combination of both have reported neutral to negative effects on growth performance and feed utilization in carnivorous fish. Indeed, the mixture of red macroalgae species (*Pyropia columbina* and *Gracilaria chilensis*) included up to 1.0% in diets for Atlantic salmon (77) and *Fucus* sp., *Gracilaria* sp., and *Ulva* sp. at 7.5% in European seabass (31, 52, 78) diets did not affect growth performance and feed intake. Similarly, no effect on growth performance and feed utilization was observed on meager fed 10% *Nannochloropsis gaditana*, *Tisochrysis lutea*, *Rhodomonas lens*, and *Isochrysis galbana* (79), and red sea bream fed 25% *Nannochloropsis* sp. and *Schizochytrium* sp. or 45% *Nannochloropsis* sp., *Chlorella* sp., and *Schizochytrium* sp. (80). On the other hand, the inclusion of *Nannochloropsis* sp. and *Isochrysis* sp. up to 11.9% reduced the feed intake and growth performance of Atlantic cod (81) and *Schizochytrium limacinum* and *N. oceanica* supplementation up to 17% reduced rainbow trout growth performance (82). The negative impact of microalgae blends was suggested to be due to their low palatability (81). The combination of macroalgae (*G. gracilis*) and microalgae (*N. oceanica*) species included at 30% did not affect European seabass growth performance or feed utilization (54).

Effects of dietary algae inclusion are species-specific with ideal inclusion levels varying with algae species and fish species (42, 72). In general, carnivorous fish, such as European seabass, digest algae recalcitrant cell walls more poorly than herbivorous fish due to the shorter intestine, the main organ for digestion and absorption (83). However, in the present study, DM, OM, CP, total lipids, GE, and most FA ADC were higher in diets with algae blend supplementation. These results contrast with the consistently reduced digestibility reported in the literature with algae inclusion, which has been attributed to algae cell walls complex polysaccharides that can resist enzymatic degradation in the stomach and small intestine of monogastric animals (84) and reduce the availability of intracellular nutrients (29, 54, 85, 86), namely ulvans in *Ulva* sp., carrageenans in *Gracilaria* sp. (16), cellulosic polymers and glucosamine, a chitin-like glycan, in *Chlorella* sp. (87), and algaenans (outer layer) and cellulosic polymers (inner layer) in *Nannochloropsis* sp. (88). We hypothesize that the ADC improvement observed with algae blend inclusion may be related to the plant-based reference diet used in this study. Experimental diets were formulated to include the algae blend at the expense of wheat gluten and whole peas, keeping constant the fishmeal and fish oil levels across diets. Whole peas were the main ingredient replaced by the algae blend (11% in Algae2, 24% in Algae4, and 32% in Algae 6). Peas (*Pisum sativum*) are moderate sources of protein (*c.a.* 22% DM basis) with low sulfur-containing amino acids, high polysaccharides, and low lipid content, but also contain antinutritional factors such as tannins, phytic acid, saponins, and trypsin inhibitor activities (89). Thus, we hypothesize that the lower digestibility observed in the control group (Algae0) with a higher pea content may be due to antinutritional factors, which may have affected the digestion and absorption of nutrients, and consequently growth performance and body composition of *D. labrax* juveniles. Gouveia and Davies (90) found that whole pea meal inclusion at 20% and 40% (DM basis) had no negative effect on palatability, feed intake, and growth performance of European seabass juveniles, but reduced carbohydrates and energy digestibility. The lower digestibility may result from the complex matrix of highly digestible non-structural polysaccharides (starch) and low digestible structural polysaccharides (fiber) of whole peas, which was suggested to limit the nutrient digestion and assimilation in rainbow trout (91). Moreover, the processing of whole peas, as peeling and extrusion, can reduce or even eliminate the antinutritional factors that may compromise feed intake and growth of fish (92).

The morphological structure of the intestine is considered a biomarker of the nutritional and physiological status, with changes related to altered nutrient digestibility (93). In the present study, the algae blend promoted villus length and width and anterior intestinal absorption area, which suggests an enhanced ability to absorb nutrients. This can at least partially explain the greater digestibility of algae-supplemented diets and consequent better feed utilization and growth of *D. labrax* juveniles. In contrast, previous studies reported no effects or even a reduction of intestinal area, or villus length and width in carnivorous fish fed diets supplemented with individual *Ulva* sp., *Gracilaria* sp., *Chlorella* sp., or *Nannochloropsis* sp. (41, 54, 94, 95), or their mixtures (54, 79, 80). Fish fed the control diet (Algae0) had the lowest number of goblet cells. These mucin-producing cells produce gel-like layers that protect epithelial mucosa, facilitate digesta transport, and protect against bacterial invasion (96). Two main subtypes of mucins are produced along the gastrointestinal tract, neutral, and acidic mucins; the former is related to digestive and absorptive processes (97) and the latter to protection against bacterial translocation (96). The tendency for neutral mucins to increase further support enhanced nutrient digestion and absorption in fish fed diets supplemented with algae blend compared to the control diet. Algae blend did not affect submucosa and lamina propria cellularity and inflammatory infiltrates, while increased submucosa and lamina propria width compared to fish fed with no algae. Further studies are needed to assess the algae blend impact on posterior intestine morphology that best relates to inflammatory processes and microbiota abundance and diversity.

Algae blend dietary inclusion had no negative impact on fish protein retention efficiency or whole-body and muscle protein content. Conversely, lipid and energy retention efficiency were promoted, which was reflected in a higher whole-body lipid and energy content of fish fed algae blend. The most marked effect of algae blend supplementation was observed on body lipid content, which gradually increased with algae inclusion levels compared to the control. Lipid metabolism, including whole body lipid

deposition and partitioning pattern, of carnivorous fish has been shown to be related to dietary energy intake and affected by dietary protein sources (marine *vs*. vegetable) that regulate lipogenic enzyme expressions and activities (98–100). In our study, a general increase in lipid deposition was observed in a fish fed algae blend for 12 weeks, with increased body and muscle lipid content and HSI. These findings are in line with the observed improvement in the digestibility of algae blend-supplemented diets and suggest the absence of bioactive compounds with lipotropic activity in the algae blend, thus contrasting with the findings of Tulli et al. (101) and suggestion of the presence of algae bioactive compounds with lipotropic activity.

Fish is the most important source of *n*-3 LC-PUFA EPA and DHA in the human diet (102). Although European seabass, like other marine finfish species, has the enzymatic ability for endogenous LC-PUFA biosynthesis, the low activity of enzymes involved in the desaturation/elongation pathway hampers the production of EPA and DHA from the C18 fatty acid precursor (α-linolenic acid; C18:3 *n*-3) at rates that meet physiological demands (103, 104). Thus, marine fish depend on dietary supplies of EPA and DHA to fulfill their essential *n*-3 PUFA requirements (102). Algae, particularly microalgae, may constitute alternative sustainable sources of *n*-3 LC-PUFA, although marked differences in the fatty acids profile are found among and within algae species (105). In addition, we hypothesize that the bioactive compounds present in algae can modulate the lipid metabolism of fish fed algae-supplemented diets, and prevent dietary fatty acids oxidation, with a putative impact on fish nutritional value and consumers' health.

In the present study, the experimental diets were formulated to include graded levels of algae blend at a constant fish oil content, thus ensuring high levels ($>30$ g kg$^{-1}$ DM basis) of EPA and DHA. Dietary algae blend inclusion was found to alter the dorsal muscle fatty acids profile, mainly by promoting MUFA and reducing PUFA proportion while total SFA proportion remained unaffected. As the fatty acids profile and content in European seabass muscle have been reported to reflect dietary fatty acids (106–108), a stepwise increase in α-linolenic (C18:3 *n*-3), stearidonic (C18:4 *n*-3), arachidonic (C20:4 *n*-6), EPA, docosapentaenoic (C22:5 *n*-3) acids, and total *n*-3 PUFA and a decrease in linoleic acid (C18:2 *n*-6), DHA, total *n*-6 PUFA, and *n*-6/*n*-3 ratio were expected in the muscle mirroring the diet fatty acids profile. However, only linoleic acid, DHA, and total PUFA *n*-6 proportion and *n*-6/*n*-3 ratio followed the expected pattern, while most individual *n*-3 PUFA and all *n*-6 PUFA proportion decreased in the dorsal muscle. These results may suggest a potential for the algae blend to modulate the lipid metabolism of *D. labrax* juveniles through selective retention or catabolism of specific fatty acids. The observed decrease in linoleic and α-linolenic acids proportions in the muscle of fish fed algae supplemented diets is in agreement with previous reports in microalgae-supplemented diets for rainbow trout (82) and turbot (33), suggesting that these C18 PUFA may have been selectively catabolized. On the other hand, dietary algae blend supplementation up to 6% had no negative effect on muscle EPA, which may suggest a preferential deposition and retention of this essential fatty acid in the muscle of European seabass juveniles. The concomitant effect of the algae blend on muscle EPA and DHA proportion contrasts with previous studies that reported DHA selective deposition and retention in the flesh of marine fish species, including European seabass (51, 107), as a result of the high specificity transferases and low catabolism of DHA, whereas EPA is often selectively catabolized by β-oxidation (82, 107). The observed modifications in the muscle fatty acids profile may be due to the high PUFA content of experimental diets provided by fish oil and algae lipids, which may have partially suppressed *de novo* fatty acid synthesis (109) and thus affected the lipid metabolism of juvenile *D. labrax*. Further studies focused on intermediary metabolism are needed to clarify this point. Of particular importance is that the algae blend improved overall muscle fatty acid retention in these fish, resulting in an increased EPA and DHA (EPA+DHA) content (mg g$^{-1}$ wet weight) to values well above the recommended 500 mg EPA+DHA per day to prevent coronary heart disease (110). Consumers would have to ingest 113 g of Algae0 fed or only 74.2 g of Algae6 fed European seabass filets. Seabass muscle lipid quality indices provide additional information on the effects of dietary algae blends on the flesh's nutritional and functional value. Atherogenicity and thrombogenicity indices are related to the risk of atherosclerosis and thrombosis, and the h/H ratio to cholesterol metabolism; lower indices and higher ratio relating to coronary health promotion (71, 111). Although dietary algae blend supplementation promoted AI and TI and reduced h/H ratio of seabass muscle, all values obtained were within the range considered to exert potential cardiovascular promoting effects (71, 111, 112). But a longer-term study should be carried out until the fish reach a commercial size to confirm the full potential of the algal blend in aquafeeds.

The skin color of the fish is of utmost importance for consumer acceptance (113). European seabass is appreciated by consumers for its white flesh, mild flavor, and low-fat content (114). In the present study, dietary algae blend supplementation altered the skin pigmentation of juvenile European seabass to a darker and less greenish color than observed in fish fed Algae0. While significant, the changes in skin pigmentation were small and mostly imperceptible to the naked eye. However, our results contrast with a more greenish skin of European seabass fed *T. suecica* (101) and *Isochrysis* sp. (108), and with the absence of effects of *G. vermiculophyla*. and *N. oceanica* supplementation, individually or as a mixture (54). In the present study, the dorsal filet color of fish fed control diet was more yellowish than those fed algae blend diets. This result agrees with the observation of Grigorakis (112) that a higher lipid content promotes a whiter color, as the muscle lipid content increased by 41.9%, 54.7%, and 66.0% with 2%, 4%, and 6% algae blend inclusion, respectively. The less yellowish color observed here may suggest an enhanced acceptance of European seabass juveniles fed up to 6% algae blend inclusion by consumers. However, these results were obtained in juvenile fish, and further studies are needed to assess the effects on pigmentation of commercial-sized fish and on consumers preference.

The fish texture is an important attribute for assessing quality, freshness, and palatability (115). A firmer texture is preferred for consumers and industry as it is considered an indicator of freshness (116), and soft filets pose difficulties to the fish processing industry (117). Algae blend supplementation reduced muscle hardness and improved adhesiveness of European seabass juveniles, compared

to fish fed Algae0. These results suggest that the algae blend diminished the texture of seabass muscle, by presenting softer traits. The softer texture of algae-fed seabass muscle was associated with lower moisture and higher lipid content. The intramuscular lipid content is considered to enhance the fish flavor and provide a smoother, juicier mouthfeel, thereby improving muscle juiciness (112). Improved juiciness may counteract the softer texture of algae blend-fed European sea juveniles. Thus, longer feeding trials and a sensory panel evaluation should be carried out to fully assess the impact of algae blend supplementation on texture traits.

## 5. Conclusion

Supplementation of *Ulva* sp., *G. gracilis*, *C. vulgaris,* and *N. oceanica* blend up to 6% to a commercial-type plant-based diet significantly improved the digestibility and feed utilization of diets as well as anterior intestine absorption area, feed intake, and growth performance of European seabass juveniles. Muscle nutritional value and quality were also improved by algae blend supplementation. Of particular importance is the increase in essential fatty acids (EPA+DHA) content, which allows for achieving daily intake recommendations for EPA and DHA even with lower consumption of fish.

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

## Ethics statement

The animal study was reviewed and approved by Animal Welfare and Ethics Body of CIIMAR (ORBEA-CIIMAR 06-2016) and licensed by the Portuguese Veterinary Authority (1005/92, DGAV-Portugal).

## Author contributions

MM, LV, AF, HA, and JS conceived and designed the study. CM, OP, TS, and MF conducted the research and performed the analyses. CD-M, AC, AA, AF, LV, and MM participated in the study coordination. CM and MM drafted the manuscript. All authors contributed to the manuscript revision, and read and approved the submitted version.

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## Conflict of interest

HA and JS are employed by the companies ALGAplus and Allmicroalgae, respectively, and CM 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.

## Publisher's note



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1165343/full#supplementary-material

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# Effects of different dietary lipid sources (perilla, fish, and soybean oils) on growth, lipid metabolism, antioxidant, and immune status in Chinese giant salamander (*Andrias davidianus*)

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**Introduction:** Perilla oil is a natural vegetable oil rich in alpha-linolenic acid extracted from Perilla seeds. Fish oil is a commonly used animal fat containing a large amount of n-3 polyunsaturated fatty acids. Soybean oil is a commonly used vegetable oil rich in linoleic acid. This study aimed to determine whether perilla, fish, or soybean oil can be used as a lipid source in the diet of the Chinese giant salamander (*Andrias davidianus*).

**Methods:** Three isonitrogenous and isolipidic diets were created by adding 3.0% each of soybean oil (SO), fish oil (FO), and perilla seed oil (PSO). Eight adolescent giant salamanders were fed in rectangular tanks for 98 days with a total of 96 giant salamanders (mean body weight = 75.20g) and were fed an experimental diet until satiation.

**Results and Discussion:** The viscerosomatic index and crude lipid content of muscle were found to be considerably lower and the muscle crude protein was substantially higher in the PSO group than in the other groups ($P<0.05$). Linoleic acid levels were highest in SO, eicosapentaenoic acid and docosahexaenoic acid levels were highest in FO, and α-linolenic acid levels were highest in PSO in *A. davidianus* muscle tissue ($P<0.05$). The results of study indicate that the PSO diet can significantly increase the total antioxidative capabilities in the liver and intestine, by elevating the activities of total superoxide dismutase, carnitine transferase-1, and acetyl-CoA carboxylase in the liver. Meanwhile, the immunoglobulin M and high-density lipoprotein cholesterol levels were higher in the blood ($P<0.05$). Furthermore, it significantly reduced hepatic malondialdehyde, plasma endotoxin, D-lactic acid, and total cholesterol levels, and plasma alkaline phosphatase, diamine oxidase, aspartate transaminase, and alanine transaminase activities ($P<0.05$). Therefore, perilla, fish, and soybean oils can be used as single lipid sources for *A. davidianus* with respect to growth

performance. On the other hand, perilla oil can enhance crude protein content of muscle, increase Immunoglobulin M(IgM) content and Alkaline phosphatase (AKP) enzyme activity, promote lipid metabolism, and maintain the health of the liver and intestine in *A. davidianus*.

KEYWORDS

***Andrias davidianus*, antioxidant, fatty acid composition, growth performance, immunity, perilla oil**

# 1 Introduction

Lipids are involved in several vital physiological activities, including providing energy for biological activity and essential fatty acids, building tissue, functioning as a carrier of lipid-soluble vitamins, and being a basic nutrient for animal growth (Segatto & Pallottini, 2020). An appropriate lipid source in the diet will help in saving protein, increasing growth performance and feed utilization efficiency, and managing immune and lipid metabolism in farm animals (Trichet, 2010).

Perilla seed oil is a natural oil produced from ripe perilla seeds and is rich in unsaturated fatty acids. Linoleic acid (C18:3n-3) is its most important component, accounting for more than 50% of the total content. PSO currently contains the most α-linolenic acid among all natural vegetable oils (Dhyani et al., 2019; Zhao et al., 2021), An important fatty acid found in freshwater fish. Desaturation and carbon chain extension can be used to transform it into long-chain, highly-unsaturated fatty acids. It is also important to the physiological, metabolic, and cytological processes in fish (Kanazawa et al., 1979). Perilla oil has great potential as a high-quality dietary oil source.

Fish oil is high in n-3 polyunsaturated fatty acids (such as eicosapentaenoic acid, EPA, and docosahexaenoic acid, DHA) (Ghasemi Fard et al., 2018), which promote the growth of aquaculture animals, improve muscle quality, boost immunity, etc (Sargent et al., 1999). It is one of the most important lipid sources in the aquatic feed sector (Peng et al., 2014). Soybean oil contains the highest amount (up to 57%) of linoleic acid (C18:2n-6), as compared to other natural vegetable oils (Ivanov et al., 2010). Many studies have proved that linoleic acid is one of the essential fatty acids of aquatic animals. Soybean oil, as one of the vegetable oils rich in linoleic acid, is widely used in aquatic animal feed. Like the shortfin corvina (*Cynoscion parvipinnis*), Nile tilapia (*Oreochromis niloticus*), largemouth bass (*Micropterus salmoides)* and other aquatic animals (Gonzalez-Felix et al., 2016; Godoy et al., 2019; Chen et al., 2020).

Giant salamander is a carnivorous, cold-water amphibian widely distributed in mountainous areas of China. Based on successful artificial breeding and feeding, our laboratory pioneered its special compound feed. Its tender meat, and high nutritional value, known as “ginseng in water”. It is a national second class protected aquatic wild animal, and also a key development species of agricultural industrialization and characteristic agriculture. (Wen-Bo et al., 2021) Chinese giant salamander has a high economic value, and has a wide range of development and utilization prospects in food, health care, medicine, ornamental and other aspects, so it is quite concerned by the community (Deng et al., 2019; Jiang et al., 2021). Currently, there has been a breakthrough in the availability and breeding of *A. davidianus* progeny; however, feed supply issues persist, and few studies have been conducted (Li et al., 2020).

As a protected animal, the Chinese giant salamander has achieved effective conservation and sustainable use of resources because of artificial breeding. With the continuous expansion of the scale of farming, the increase in breeding costs and the decline in commodity prices, The need for compound feed development and nutritional research has become urgent. To further optimize the feed formulation,this study aimed to determine perilla seed oil, fish oil and soybean oil the growth, lipid metabolism, oxidation state, and immunity of *Andrias davidianus.*

# 2 Material and methods

## 2.1 Experimental diet

The diets in this trial were prepared with fish and chicken meals as fundamental raw materials, in accordance with the nutritional demands of *A. davidianus* (Wang et al., 2020). PSO, FO, and SO constitute three isonitrogenous and isolipidic diet groups prepared by adding perilla, fish, or soybean oil as the only lipid source, respectively. A mixed pellet-making device crushed the raw materials using a 40-mesh screen and pelletized them into 4 mm-diameter particles. They were air-dried before being packaged, wrapped in double-layer plastic bags, and stored at -20°C until further use. The details of the experimental feed mix and fundamental nutritional makeup are listed in Table 1. Table 2 shows the fatty acid makeup of the various oil sources and diets.

## 2.2 Experimental procedure and samples collection

Juvenile *A. davidianus* were procured from the Luyuan Giant Salamander Farm in Hanzhong, Shaanxi Province, China (Shaanxi species, second generation, and in compliance with relevant

TABLE 1 Formulation and nutrient composition of the diets (dry matter basis, g/kg).

| Ingredients | Diet |
|---|---|
| Fish meal | 400 |
| Chicken meal | 200 |
| Compound protein powder[a] | 172 |
| α-Pregelatinized starch | 100 |
| Maltodextrin | 20 |
| Oil | 30 |
| $Ca(H_2PO_4)_2$ | 15 |
| Premix[b] | 63 |
| Proximate composition | |
| Moisture (%) | 8.2 |
| Crude protein (%) | 50.8 |
| Crude lipid (%) | 9.6 |
| Crude ash (%) | 12.4 |
| Gross energy (MJ/Kg) | 19.13 |

Fish oil, soybean oil, and perilla oil were from Xingye Oil Co., Ltd., Zhejiang, China, Red Dragonfly Oil Co., Ltd., Chongqing, China, and Zhongxian Xiangnan Agricultural Development Co., Ltd., Chongqing, China, respectively.The remaining feed ingredients came from Dafa Feed Co., Ltd., Chongqing, China.
[a]Compound protein powder (Confidential Agreement).
[b]Premix contains multi-vitamins, complex minerals, functional additives and carriers, multi-vitamins, and complex minerals suitable for carnivorous fishes (Coutinho et al., 2012).

breeding regulations), and allowed to rest for 2 days until the formal experiment. They were sterilized using 1% NaCl solution for 5 min and then acclimated with basic feed (Diet FO) in an indoor freshwater tank for 2 weeks. A total of 96 healthy *A. davidianus* with an initial body weight of 75.20 ± 1.18 g were randomly divided into three experimental groups. Each group was further divided into four replicates, with eight salamanders in each. They were reared in blue plastic square boxes (70×45×17.5 cm) from the Southwest University Aquaculture System of College of Fisheries. The water was thoroughly aerated and maintained at a depth of 5 cm. The water was refilled every day in the morning and evening. During the transient period, A. davidianus were fed only once a day at 17:00 until there were residual bait to determine the satiety level of A. davidianus. During the formal experiment, feeding was done for two days (satiety) and stopped for one day, as they need longer time to digest the food.Every day, residual baits were collected, and their weights were recorded. The *A. davidianus* were grown and fed the diets in darkness during the 12-week study, with water at 19-20°C, 6.5–7.5 pH, 5.5-6.0 mg/L of dissolved oxygen levels, and less than 0.1 mg/L of total ammonia levels.

As determined by our laboratory, the intestinal emptying time of A. davidianus was 4 days, therefore, *A. davidianus* were fasted for four days at the end of the route before sampling (Li et al., 2020). Only is presented a sampling at the end of the trial, 8 A. *davidianus* were randomly selected and anesthetized with MS-222 (75 mg/L; Sigma, St Louis, MO, USA) to collect blood from the abdominal artery using heparinized sterile syringes in each replicate. Blood samples were centrifuged for 10 minutes at 4°C and 1970xg (centrifuge model 5430 R; Eppendorf, Hamburg, Germany), and plasma was extracted and stored at −80°C until further analysis. Another 12 tails after anesthesia are taken to remove viscera.The liver was then excised. The stomach and intestine contents were emptied out and rinsed with phosphate-buffered saline (PBS) (0.064 mol/L, pH 7.4). The tissues were immediately frozen in liquid nitrogen and stored in a refrigerator at −80°C to evaluate tissue enzyme activity. Dorsal samples of the liver and muscle samples were obtained and stored at −20°C until future investigation. All tests were performed in accordance with China's standard protocol for the care and use of experimental animals. This study was approved by the Animal Ethics Committee of the Southwest University.

## 2.3 Growth performance and morphologic indices

The growth parameters were calculated as follows:

Weight gain rate (WGR, %)=100×(final body weight, FBW-initial body weight, IBW)/initial body weight

Specific growth rate (SGR, %)=100×(*Ln* FBW-*Ln* IBW)/number of feeding days

Feed intake (FI, g/tail)=total feed intake (g)/number of *A.davidianus*

Feed conversion ratio (FCR)=dry diet intakefeed (g)/wet weight gain (g)

Survival (%)=100×(final number of *A.davidianus*)/(initial number of *A.davidianus*).

Viscerosomatic Index (VSI, %)=(weight of viscera/FBW)×100

Hepatosomatic Index (HSI, %)=(weight of liver/FBW)×100

Condition Factor (CF, %)=(weight of *A.davidianus*/body length$^3$)×100

## 2.4 Determination of the nutrient composition of diets and muscle

Crude protein, lipid, ash, and moisture contents of diets and muscle were determined according to the methods described by the Association of Official Analytical Chemists, 2005 (AOAC) (Feldsine et al., 2002). Moisture content was determined by drying to constant weight at 105°C. Crude protein content (N×6.25) was determined by the Kjeldahl method using a nitrogen analyzer (KDN-812, Shanghai, China) after acid digestion. Lipid content was determined using petroleum ether (BP, at 30-60°C for 3 h) and a fat analyzer (SZC-D, Shanghai, China). Fatty acid composition of lipid sources, diets, and muscle was determined with the help of Baishilu Biotechnology Co., Ltd, Guangzhou, China.

TABLE 2 Fatty acid composition of lipid sources and the experimental diets (total fatty acids, %).

| Fatty acids | Lipid sources | | | Diets | | |
|---|---|---|---|---|---|---|
| | soybean oil | fish oil | perilla oil | SO | FO | PSO |
| C14:0 | 0.08 | 6.77 | — | 2.42 (0.23) | 4.33 (0.42) | 2.68 (0.26) |
| C16:0 | 10.94 | 20.93 | 6.88 | 17.52 (1.68) | 21.74 (2.09) | 16.72 (1.61) |
| C18:0 | 3.78 | 3.89 | 1.84 | 5.07 (0.49) | 5.46 (0.52) | 4.34 (0.42) |
| C24:0 | 0.00 | 0.10 | — | 0.13 (0.01) | 0.13 (0.01) | 0.09 (0.01) |
| ΣSFA | 14.80 | 31.69 | 8.72 | 25.13 (2.41) | 31.66 (3.04) | 23.82 (2.29) |
| C16:1n-7 | 0.25 | 8.06 | 0.18 | 3.43 (0.33) | 5.31 (0.51) | 3.76 (0.36) |
| C18:1n-9 | 24.85 | 17.87 | 15.90 | 24.08 (2.31) | 22.63 (2.17) | 24.82 (2.38) |
| C24:1 | 0.13 | 12.16 | — | 0.08 (0.01) | 0.24 (0.02) | 0.09 (0.01) |
| ΣMUFA | 25.23 | 38.09 | 16.08 | 27.59 (2.65) | 28.18 (2.71) | 28.66 (2.75) |
| C18:2n-6 | 51.66 | 3.20 | 15.10 | 31.56 (3.03) | 15.42 (1.48) | 15.10 (1.45) |
| C18:3n-3 | 6.92 | 4.38 | 64.10 | 3.41 (0.33) | 1.69 (0.16) | 21.38 (2.05) |
| C18:3n-6 | 0.42 | 0.98 | — | 0.04 (0.004) | 0.07 (0.01) | 0.02 (0.002) |
| C20:4n-6 | 0.15 | 2.07 | — | 0.63 (0.06) | 1.06 (0.10) | 0.02 (0.002) |
| C20:5n-3 | 0.55 | 7.05 | — | 3.11 (0.30) | 5.32 (0.51) | 3.49 (0.36) |
| C22:6n-3 | 0.06 | 12.14 | — | 2.53 (0.24) | 6.94 (0.67) | 3.01 (0.29) |
| ΣPUFA | 59.76 | 29.82 | 79.20 | 41.26 (3.96) | 30.49 (2.93) | 43.02 (4.13) |
| ΣHUFA | 0.76 | 21.26 | — | 6.26 (0.60) | 13.31 (1.28) | 6.52 (0.63) |
| EPA+DHA | 0.61 | 19.19 | — | 5.64 (0.52) | 12.26 (1.18) | 6.50 (0.63) |
| Σn3 | 7.53 | 23.57 | 64.10 | 9.04 (0.87) | 13.95 (1.34) | 27.88 (2.68) |
| Σn6 | 52.23 | 6.25 | 15.10 | 32.22 (3.09) | 16.54 (1.59) | 15.14 (1.45) |
| Σn3/Σn6 | 0.14 | 3.77 | 4.25 | 0.28 (0.28) | 0.84 (0.84) | 1.84 (1.84) |

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; HUFA, highly unsaturated fatty acids; Σn3, total n3 polyunsaturated fatty acids; Σn6, total n6 polyunsaturated fatty acids. The composition of fatty acids in the diet is shown in parentheses, and the content in extracted fat is outside the parentheses.

## 2.5 Determination of plasma biochemical indexes

The activities of diamine oxidase (DAO), endotoxin (ET), D-lactic acid (D-LA), and immunoglobulin M (IgM) were measured by double antibody sandwich ELISA method(ELISA kits of Shanghai Enzyme-linked Biotechnology). Plasma aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) levels were measured by Multifunctional enzyme marker (Multiskan Sky, China) using a commercial test kit (Nanjing Jiancheng Institute of Biological Engineering) according to the manufacturer's instructions.

## 2.6 Determination of antioxidant enzymes in liver, stomach, and intestinal tissues

Total protein(TP) was determined by coomassie blue staining method (Deng et al., 2013). Total antioxidant capacity (T-AOC) and total superoxide dismutase (T-SOD) of liver, stomach and intestine were measured according to the method described by (Wei et al., 2010); malondialdehyde (MDA) levels were measured according to the method of (Loh et al., 2010), all using a commercial kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The unit of TAOC is (U/mgprot), (U) is a unit of total antioxidant capacity (U) per milligram of tissue protein per minute at 37°C, which increases the absorbance (OD) value of the reaction system by 0.01.Carnitine palmitoyltransferase-1 (CPT-1), and acetyl-CoA carboxylase (ACC) activities in liver were detected by enzyme-linked immunosorbent assay (ELISA kits of Shanghai Enzyme-linked Biotechnology).

## 2.7 Statistical analysis

All data were subjected to a one-way analysis of variance (ANOVA) after Duncan's multiple range test. $P<0.05$ was considered statistically significant, and the results were expressed as mean ± standard deviation (SD) of quadruplicate tanks. All

statistical analyses were performed using SPSS 22.0 for Windows (v. 13.0; SPSS).

# 3 Results

## 3.1 Effects of three kinds of lipid source on growth performance and morphologic index of *A. davidianus*

The effects of the three kinds of lipid sources on the growth performance and morphologic index of *A. davidianus* are shown in Table 3. FBW, WGR, SGR, FCR, HSI, CF, and the survival rate were not significantly different among treatments ($P$>0.05). The VSI of PSO was significantly lower than that of SO and FO ($P$<0.05).

## 3.2 Effects of three kinds of lipid source on composition of nutrients in muscle of *A. davidianus*

Crude protein content of muscle in PSO was significantly higher than that in SO and FO ($P$<0.05) (Table 4). Crude lipid content of muscle was significantly lower than that of SO but was not significantly different between PSO and FO ($P$>0.05). The moisture content in muscles was not significantly different between the groups ($P$>0.05).

## 3.3 Effects of three kinds of lipid source on muscle fatty acid of *A. davidianus*

The effects of the three lipid sources on the muscle fatty acids of *A. davidianus* are shown in Table 5. The monounsaturated fatty acid(NUFA) content in the PSO group was significantly higher than that in the FO group (P<0.05), but the difference between the SO and FO groups was not significant. The polyunsaturated fatty acid content in SO was higher than that in PSO and FO (P<0.05). The level of C18:2n-6 in SO was significantly higher than that in the other groups (P<0.05), while that of C18:3n-3 in PSO was significantly higher than that in SO and FO (P<0.05), indicating improvement of 235.59% and 318.31%, respectively. EPA and DHA were the highest in FO, EPA was significantly higher in FO than in other groups (P<0.05), and DHA was significantly higher in FO than in PSO (P<0.05). However, the difference between FO and SO was not significant (P>0.05). The n-3 and n-6 polyunsaturated fatty acids (PUFAs) were higher in PSO and SO than in the other two groups, respectively (P<0.05). Regression and correlation analyses of muscle fatty acid (Yi) and the corresponding dietary fatty acid

TABLE 3 Effects of three kinds of lipid source on growth performance and morphologic indices of *Andrias davidianus*.

| Diets | SO | FO | PSO |
|---|---|---|---|
| IBW (g) | 76.10 ± 0.70 | 74.83 ± 0.32 | 74.33 ± 1.63 |
| FBW (g) | 140.25 ± 3.32 | 143.15 ± 4.31 | 136.13 ± 4.38 |
| WGR (%) | 84.35 ± 2.1 | 91.64 ± 5.61 | 83.10 ± 2.45 |
| SGR (%/day) | 0.63 ± 0.01 | 0.66 ± 0.03 | 0.62 ± 0.02 |
| FCR | 0.51 ± 0.02 | 0.49 ± 0.03 | 0.51 ± 0.03 |
| Survival (%) | 100 | 100 | 100 |
| Morphologic indices | | | |
| HSI (%) | 3.97 ± 0.47 | 4.23 ± 0.47 | 3.97 ± 0.46 |
| VSI (%) | 13.36 ± 1.39$^{b}$ | 12.90 ± 1.02$^{b}$ | 11.04 ± 0.98$^{a}$ |
| CF (g/cm$^{3}$) | 0.47 ± 0.04 | 0.45 ± 0.05 | 0.47 ± 0.05 |

Values are presented as mean ± SD of the four replicates. Mean values in the same row with different superscripts are significantly different (P<0.05) based on Post Hoc test.
CF, Condition factor; FBW, final body weight; FCR, Feed conversion ratio; FI, feed intake; HSI, Hepatosomatic index; IBW, initial body weight; SGR, specific growth rate; VSI, Viscerosomatic index; WGR, weight gain rate.

TABLE 4 Effects of three kinds of lipid source on muscular composition of *A. davidianus* (fresh).

| Diets | SO | FO | PSO |
|---|---|---|---|
| moisture/% | 83.18 ± 0.58 | 83.27 ± 0.47 | 82.97 ± 0.81 |
| crude protein/% | 14.91 ± 0.07$^{a}$ | 14.91 ± 0.03$^{a}$ | 15.25 ± 0.01$^{b}$ |
| crude lipid/% | 0.70 ± 0.04$^{b}$ | 0.61 ± 0.03$^{ab}$ | 0.60 ± 0.05$^{a}$ |

Values are presented as mean ± SD of the four replicates. Mean values in the same row with different superscripts are significantly different (P<0.05).

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TABLE 5 Effects of three kinds of lipid source on muscle fatty acid (total fatty acids, %) of *A. davidianus*.

| Fatty acids | Diets | | |
|---|---|---|---|
| | SO | FO | PSO |
| C14:0 | 1.19 ± 0.65 (0.008 ± 0.004) | 2.33 ± 0.02 (0.014 ± 0.000) | 1.77 ± 0.17 (0.011 ± 0.001) |
| C16:0 | 18.92 ± 0.78 (0.132 ± 0.005) | 20.91 ± 1.03 (0.128 ± 0.006) | 18.33 ± 0.62 (0.110 ± 0.004) |
| C18:0 | $8.03 \pm 0.09^{b}$ ($0.056 \pm 0.000^{b}$) | $6.78 \pm 0.15^{a}$ ($0.041 \pm 0.001^{a}$) | $8.28 \pm 0.32^{b}$ ($0.050 \pm 0.002^{b}$) |
| C24:0 | — | — | — |
| ΣSFA | 28.13 ± 0.23 (0.197 ± 0.002) | 30.01 ± 0.86 (0.183 ± 0.005) | 28.37 ± 1.10 (0.170 ± 0.007) |
| C16:1n-7 | $4.55 \pm 0.02^{a}$ ($0.032 \pm 0.000^{a}$) | $5.39 \pm 0.4^{b}$ ($0.0329 \pm 0.002^{b}$) | $4.74 \pm 0.06^{ab}$ ($0.028 \pm 0.000^{ab}$) |
| C18:1n-9 | $20.94 \pm 0.31^{b}$ ($0.147 \pm 0.002^{b}$) | $17.63 \pm 0.6^{a}$ ($0.108 \pm 0.004^{a}$) | $21.13 \pm 0.07^{b}$ ($0.127 \pm 0.000^{b}$) |
| C24:1 | — | — | — |
| ΣMUFA | $25.49 \pm 0.29^{b}$ ($0.178 \pm 0.002^{b}$) | $23.01 \pm 0.20^{a}$ ($0.140 \pm 0.001^{a}$) | $25.87 \pm 0.13^{b}$ ($0.155 \pm 0.001^{b}$) |
| C18:2n-6 | $20.01 \pm 0.95^{b}$ ($0.140 \pm 0.007^{b}$) | $14.83 \pm 0.20^{a}$ ($0.091 \pm 0.001^{a}$) | $13.92 \pm 0.18^{a}$ ($0.084 \pm 0.001^{a}$) |
| C18:3n-3 | $1.77 \pm 0.02^{a}$ ($0.012 \pm 0.000^{a}$) | $1.42 \pm 0.12^{a}$ ($0.009 \pm 0.001^{a}$) | $5.94 \pm 0.25^{b}$ ($0.036 \pm 0.002^{b}$) |
| C18:3n-6 | — | — | — |
| C20:4n-6 | 5.75 ± 0.62 (0.040 ± 0.004) | 4.90 ± 0.09 (0.030 ± 0.001) | 5.51 ± 0.45 (0.033 ± 0.003) |
| C20:5n-3 | $4.86 \pm 0.09^{a}$ ($0.034 \pm 0.001^{a}$) | $6.50 \pm 0.13^{b}$ ($0.040 \pm 0.001^{b}$) | $5.17 \pm 0.09^{a}$ ($0.031 \pm 0.001^{a}$) |
| C22:6n-3 | $7.69 \pm 0.04^{ab}$ ($0.054 \pm 0.000^{ab}$) | $8.70 \pm 0.25^{b}$ ($0.053 \pm 0.002^{b}$) | $7.35 \pm 0.42^{a}$ ($0.044 \pm 0.003^{a}$) |
| ΣPUFA | $40.06 \pm 0.23^{c}$ ($0.280 \pm 0.002^{c}$) | $36.34 \pm 0.37^{a}$ ($0.222 \pm 0.002^{a}$) | $37.87 \pm 0.54^{b}$ ($0.227 \pm 0.003^{b}$) |
| ΣHUFA | $18.29 \pm 0.75^{a}$ ($0.128 \pm 0.005^{a}$) | $20.10 \pm 0.29^{b}$ ($0.123 \pm 0.002^{b}$) | $18.02 \pm 0.12^{a}$ ($0.108 \pm 0.001^{a}$) |
| EPA+DHA | $12.55 \pm 0.13^{a}$ ($0.088 \pm 0.001^{a}$) | $15.20 \pm 0.38^{b}$ ($0.093 \pm 0.002^{b}$) | $12.51 \pm 0.33^{a}$ ($0.075 \pm 0.002^{a}$) |
| Σn3 | $14.31 \pm 0.11^{a}$ ($0.100 \pm 0.001^{a}$) | $16.62 \pm 0.26^{b}$ ($0.101 \pm 0.002^{b}$) | $18.45 \pm 0.08^{c}$ ($0.111 \pm 0.000^{c}$) |
| Σn6 | $25.75 \pm 0.34^{b}$ ($0.180 \pm 0.002^{b}$) | $19.73 \pm 0.11^{a}$ ($0.120 \pm 0.001^{a}$) | $19.42 \pm 0.62^{a}$ ($0.117 \pm 0.004^{a}$) |
| Σn3/Σn6 | $0.56 \pm 0.01^{a}$ ($0.56 \pm 0.01^{a}$) | $0.84 \pm 0.01^{b}$ ($0.84 \pm 0.01^{b}$) | $0.95 \pm 0.03^{c}$ ($0.95 \pm 0.03^{c}$) |

Values are presented as mean ± SD of the four replicates. Mean values in the same row with different superscripts are significantly different (P<0.05).
The composition of fatty acids in muscle is shown in parentheses, and the content in extracted fat of muscle is outside the parentheses.

(Xi) from each treatment showed that there was a highly significant linear correlation between muscle fatty acid composition and forage fatty acid composition in the SO and FO groups, with correlation coefficients of 0.920 and 0.962, respectively, and relatively poor in PSO (Table 6).

## 3.4 Effects of three kinds of lipid source on antioxidant ability of *A. davidianus*

The effects of the three lipid sources on the antioxidant ability of *A. davidianus* are shown in Figure 1. The activities of liver T-AOC and T-SOD in PSO were significantly higher than those in SO (P<0.05). The MDA content in FO was significantly lower than that in SO (P<0.05), but was not significantly different as compared to PSO (P>0.05). The order of T-SOD activity in gastric tissue is FO>PSO>SO, and with significant differences among treatment groups.T-AOC of gastric tissue in FO group was remarkably higher than that in SO group(P<0.05), but there was no significant difference between FO and PSO (P>0.05).The MDA content was not significantly different among the three treatment groups (P>0.05). T-AOC of intestinal tract was significantly higher in PSO than in SO (P<0.05), but not significantly different between PSO and FO (P>0.05). T-SOD activity in FO group was significantly higher than that in the other treatment groups (P<0.05). There was no significant difference in MDA content among all groups (P>0.05).

TABLE 6 Regression and correlation analysis of essential fatty acids between muscle and experimental diets.

| Diets | Related coefficient (r) | P | $R^2$ | Equation of linear regression |
|---|---|---|---|---|
| SO | 0.920 | <0.01 | 0.847 | $Y_1 = 0.644X_1 + 3.336$ |
| FO | 0.962 | <0.01 | 0.926 | $Y_2 = 0.799X_2 + 1.755$ |
| PSO | 0.746 | <0.01 | 0.558 | $Y_3 = 0.529X_3 + 4.165$ |

Values are presented as mean ± SD of the four replicates. Mean values in the same row with different superscripts are significantly different (P<0.05).

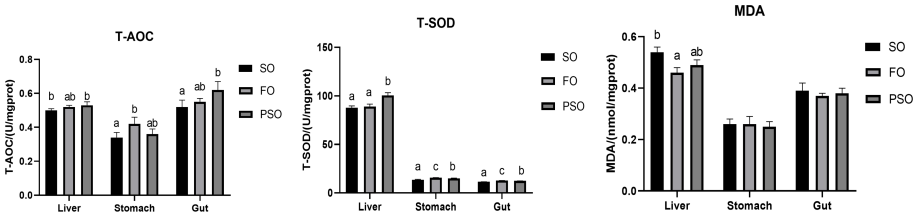


FIGURE 1
Effects of three kinds of lipid source on antioxidant ability of *A. davidianus*. Values in each column with different superscripts have significant differences (p< 0.05). T-AOC, Total antioxidant capacity; T-SOD, Total superoxide dismutase; MDA, Malondialdehyde.

## 3.5 Effects of three kinds of lipid source on immunity and liver function of *A. davidianus*

As shown in Figure 2, in plasma biochemical indices, IgM content in PSO was significantly different from that in SO and FO (P<0.05), increasing by 51.83% and 21.88%, respectively.The activity of ALP was significantly lower in PSO than that in SO (P<0.05), but was not significantly different between PSO and FO. The activity of ALT was significantly lower in PSO than that in FO (P<0.05), but was not significantly different between PSO and SO. The activity of AST was significantly lower in PSO than that in SO and FO (P<0.05).

## 3.6 Effects of three kinds of lipid source on intestinal mucosal permeability of *A. davidianus*

The content of ET and D-LA, and the activity of DAO in PSO were significantly reduced by 22.12% and 14.43%, respectively (P<0.05) (Table 7), and that of D-LA was significantly lower (P<0.05) than SO and FO by 23.27% and 11.27%, respectively (Table 7).

## 3.7 Effects of three kinds of lipid source on lipid metabolism of *A. davidianus*

The effects of the three lipid sources on *A. davidianus* lipid metabolism are shown in Table 8. In plasma biochemical indices,the TC and TG contents in FO were significantly lower than those in SO (P<0.05), but not significantly different between SO and PSO (P>0.05). HDL-C level was significantly higher in PSO than in SO (P<0.05). The LDL-C levels were not significantly different between the treatment groups (P > 0.05). In the liver, the activities of CPT-I and ACC were highest in PSO(P<0.05). In addition, the former's activity increased by 96.59% and 32.84%, and that of the latter by 59.86% and 24.94%, as compared to SO and FO, respectively (P<0.05).

# 4 Discussion

The difference between different types of lipid lies in fatty acid composition, fat structure, processing methods, etc. The lipid source selection in aquatic animal feed often depends on the

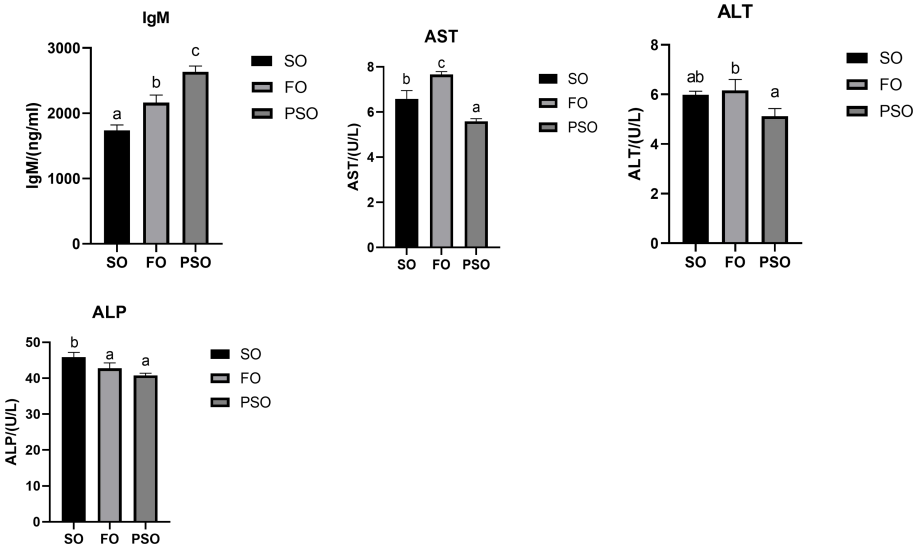


FIGURE 2
Effects of three kinds of lipid source on plasma immune indices of *A.davidianus*. Values in each column with different superscripts have significant differences (p< 0.05). IgM, Immunoglobulin M; AST, Aspartate aminotransferase; ALT, Alanine aminotransferase; ALP, Alkaline phosphatase.

TABLE 7 Effects of three kinds of lipid source on intestinal mucosal permeability of *A. davidianus*.

| Diets | SO | FO | PSO |
|---|---|---|---|
| ET/(EU/mL) | 50.55 ± 1.21$^{b}$ | 48.54 ± 2.48$^{b}$ | 42.35 ± 1.36$^{a}$ |
| DAO/(U/mL) | 27.13 ± 0.62$^{c}$ | 24.18 ± 1.02$^{b}$ | 21.13 ± 0.69$^{a}$ |
| D-LA/(μmol/mL) | 359.41 ± 9.88$^{c}$ | 306.86 ± 5.01$^{b}$ | 275.78 ± 9.44$^{a}$ |

Values are presented as mean ± SD of the four replicates. Mean values in the same row with different superscripts are significantly different (P<0.05).
ET, Endotoxin; DAO, diamine oxidase; D-LA, D-lactic acid.

utilization ability of different fatty acids.In the experiment of using different Marine lipid sources on the growth performance of Large Yellow Croaker(*Larimichthys crocea*) (Yao et al., 2022), replacing fish oil with *Schizochytrium* sp.oil(SSO) will have an inhibitory effect on appetite and growth of young large yellow croakers.In a study on the growth performance of spotted sea bass (*Lateolabrax maculatus*) using different dietary fat sources (Jin et al., 2023), compared with FO, the 16:0, 18:0, 18:1n-9, 18:2n-6 and 18:3n-3 series of fatty acids were more present in vegetable oil. The final results showed that substituting FO with vegetable oil did not have any negative effect on the growth performance of spotted sea bass. Similar results were obtained in the study (Liang et al., 2022). When coconut oil (CO), rapeseed oil (RO), linseed oil (LO) and FO were used as lipid sources, there were no significant differences in FBW, SGR, FE, survival rate, HIS and VSI of fish among all groups. This may be because LO is rich in C18:3n-3, which can be used well by freshwater fish.RO is rich in 18:1n-9 and 18:2n-6 essential fatty acids of freshwater fish, and CO is rich in medium chain fatty acids (C10:0, C12:0 and C14:0), which can regulate lipid metabolism. Therefore, replacing FO with CO, RO and LO will not affect the growth performance of *Micropterus salmoides*.In our study, the protein and lipid contents of the experimental diets were approximately the same, which all met the basic nutritional requirements of juvenile giant salamander. After the end of breeding, no obvious fatty acid deficiency symptoms and no significant difference in growth performance were found in all experimental groups. According to the fatty acids content of the three oils identified in this study, Perilla oil is rich in alpha-linolenic acid, soybean oil is rich in linoleic acid, and fish oil is rich in EPA, DHA and other highly unsaturated fatty acids. Therefore, the authors suggested that the giant salamander had the ability to convert alpha-linolenic acid (ALA) and linoleic acid (LA) into long-chain polyunsaturated fatty acids (LC-PUFA).A vital method for animals to meet their requirements of essential fatty acids is through feeding. Animals digest dietary lipids and break them down into fatty acids, which are then used to resynthesize their own fat. Dietary fatty acid composition can affect fatty acid composition in animal muscles (Ji et al., 2011; Li et al., 2016), which has also been confirmed by the present study. In this experiment, after feeding *A. davidianus* a diet containing different oils for 14 weeks, the muscle fatty acids showed a significant difference. The α-linolenic acid content of the muscles in PSO was much higher than that in the other two groups. The α-linolenic acid is an essential unsaturated fatty acid for humans, is known as vitamin F, is an important structural and metabolic regulatory substance, and can also be converted into DHA, DPA, EPA, etc. to maintain normal physiological functions of the body (Roesch et al., 2007). The muscles of *A. davidianus* that are rich in linolenic acid exhibit better quality and meet human nutritional needs well. The highest content of linoleic acid was observed in SO, and that of EPA and DHA in FO because of their possible role in the formation of cell membranes, effect on the activity of some enzymes and expression of some genes, and the organisms' preference for it to be deposited and stored. While there was a significant linear correlation between muscle fatty acid composition and dietary fatty acids in SO and FO, the correlation in PSO was poor, possibly due to the high content of α-linolenic acid in the diet. *A. davidianus* was unable to absorb and utilize the linolenic acid completely, resulting in its wastage and

TABLE 8 Effects of three kinds of lipid source on lipid metabolism of *A. davidianus*.

| Diet | SO | FO | PSO |
|---|---|---|---|
| Plasma | | | |
| TC/(mmol/L) | 1.70 ± 0.14$^{b}$ | 1.42 ± 0.13$^{a}$ | 1.58 ± 0.10$^{ab}$ |
| TG/(mmol/L) | 0.82 ± 0.06$^{b}$ | 0.62 ± 0.03$^{a}$ | 0.71 ± 0.04$^{ab}$ |
| HDL-C/(mmol/L) | 0.06 ± 0.01$^{a}$ | 0.09 ± 0.01$^{ab}$ | 0.10 ± 0.01$^{b}$ |
| LDL-C/(mmol/L) | 0.28 ± 0.01 | 0.25 ± 0.02 | 0.25 ± 0.03 |
| Liver | | | |
| CPT-1/(U/L) | 206.36 ± 14.18$^{a}$ | 305.39 ± 10.27$^{b}$ | 405.68 ± 7.55$^{c}$ |
| ACC/(U/mL) | 721.60 ± 50.35$^{a}$ | 923.30 ± 41.73$^{b}$ | 1153.54 ± 48.46$^{c}$ |

Values are presented as mean ± SD of four replicates. Mean values in the same row with different superscripts are significantly different (P<0.05).
TC, total cholesterol; TG, triglyceride; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; CPT-1, carnitine palmitoyltransferase-1; ACC, acetyl-CoA carboxylase.

requiring further analysis of its optimal amount in the diet. Overall, the difference in fatty acids in *A. davidianus* muscle was less amongst its diet groups of the three treatments, indicating that it can selectively deposit or utilize fatty acids from diets.

Perilla oil is rich in α-linolenic acid and its blood lipid-lowering effects have been previously described extensively (Winnik et al., 2011; Tsukamoto & Sugawara, 2018). It reduces 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG COA) activity, enhances acyl-coenzyme A cholesterol acyltransferase (ACAT) activity, inhibits endogenous cholesterol synthesis, and lowers blood plasma cholesterol levels by reducing triglycerides and apolipoprotein B of very low-density lipoprotein biosynthesis to reduce serum triglyceride levels. Simultaneously, the breakdown of the low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) synthesis mechanisms exhibited an efficient inhibitory effect, thereby improving the body's lipid metabolism. EPA and DHA can also reduce the synthesis of endogenous cholesterol and inhibit triglycerides, lowering the levels of arterial stiffness factor (cholesterol, triglycerides, and low-density lipoprotein). Lecithin cholesterol transfer improves lipoprotein enzyme activity, inhibition of liver endothelial lipase activity, and resistance to hardening of arterial factor (HDL) to improve lipid metabolism. In this study, the lipid-lowering effects of perilla and fish oils were also elucidated, and the lipid contents in PSO and FO were lower than those in SO. The key rate-limiting enzymes of fatty acid degradation (CPT-1) and synthesis (ACC) in the liver increased as SO>FO> PSO. However, the rate of increase of CPT-1 was significantly higher than that of ACC, suggesting that the liver lipid deposition in FO and PSO was lower than that in SO, and that perilla oil and fish oil can reduce liver fat deposition and improve liver health, which is also reflected in the classic assessment indicators of liver health (plasma AST and ALT activities). In addition, the muscle fat measurement results showed that the fish and perilla oils also reduced the amount of fat deposited in muscle tissue.

Lipids, particularly PUFAs, contain multiple double bonds and are easily oxidized by free radicals to produce several harmful substances (Tocher et al., 2002). Studies have shown that superoxide dismutase(SOD) and catalase (CAT) are two important free radical scavenging enzymes in the cellular antioxidant system. The body can reduce the damage caused by peroxidation by scavenging the oxygen free radicals produced by SOD, CAT and other enzymes (Mourente et al., 2007). The addition of different types of fatty acids to the diet has different effects on the antioxidant system of the body. Perilla oil had the highest content of PUFAs amongst the three oils used in this study, followed by soybean oil, with the lowest content in fish oil. However, according to the antioxidant indices obtained from the tissues of the three groups in this study, the antioxidant capacity of PSO was the highest, followed by FO, and that of SO was relatively poor. This may be due to the freshness of the oil used in this experiment and the addition of sufficient antioxidants to the feed, ensuring that the level of oxidation in the feed was low before it was ingested by A. davidianus.

Additionally, the antioxidant activity of lipids is closely related to the position of the double bond. A previous study has shown that n-3 PUFA can react with oxidative free radicals in the organism and protect it from oxidative damage (Collison et al., 2005). Chen also reached a similar conclusion in the study of black carp (Mylopharyngodon piceus)(Yanting et al., 2022). The experimental results show that the diet with rapeseed oil as fat source is rich in n-3 LC-PUFA, which can inhibit the production of H2O2 in cells through the nuclear factor-related factor 2 (Nrf2) pathway, so as to improve the antioxidant capacity of black carp.In this experiment, the percentage of n-3 PUFA in perilla oil was the highest due to the abundance of α-linolenic acid, fish oil was also rich in EPA, DHA, and other n-3 PUFA, and the linoleic acid in soybean oil was n-6 PUFA, which is a possible reason for the difference in antioxidant performance among the three treatment groups of A. davidianus.The immunity of cultured animals is affected by the type, dosage, and ratio of dietary fatty acids. In fish, appropriate levels of PUFAs, especially n-3 PUFA, can significantly improve immunity (Wang et al., 2006). The study of Ferreira (Ferreira et al., 2015) showed that linseed oil(LO), which is rich in n-3 PUFA, had immunomodulatory effects on juvenile Nile tilapia when added to the low-fat diet. In addition, some studies have shown that EPA and DHA in diets can also significantly affect specific and non-specific immunity of fish (Puangkaew et al., 2004) and the expression of immune-related genes (Zuo et al., 2012). IgM is an immunoglobulin present in body fluids, which plays an important role in humoral immune response (Awad et al., 2015). In this experiment, the level of IgM in PSO group was the highest, followed by FO group, and the lowest in SO group, which was the same as the content trend of n-3PUFA in the experimental feed group. Therefore, we suggest that n-3PUFA can enhance the immune function of A. davidianus, and the ability of A. davidianus to convert α-linolenic acid into EPA and DHA may be one of the reasons for the highest IgM levels in the PSO group, but the mechanism of action needs to be further investigated.A previous study found that n-3 PUFA can reduce intestinal inflammation (Calder, 2008), promote repair of the intestinal mucosa, and improve intestinal barrier function. Plasma levels of endotoxin, diamine oxidase, and D-lactic acid are three indicators of gut barrier function, and we speculate their changes to also be related to n-3 PUFA levels in the experimental diets of this study.

## 5 Conclusion

Perilla, fish, and soybean oils can all be used as a single dietary lipid source for *A. davidianus* when growth performance is used as a reference. Perilla oil can enhance muscle quality and antioxidant capacity, boost immunity, promote lipid metabolism, and maintain liver and intestinal health as fat source of *A. davidianus* compound feed. Therefore, we strongly recommend the addition of perilla oil to their diet.

## Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

## Ethics statement

The animal study was reviewed and approved by Southwest University's Animal Ethics Committee.

## Author contributions

The authors confirm contribution to the paper as follows: LL: study conception and design. Y-HG: performed the experiment, analyzed the data, writing—original draft, writing—review and editing. J-KM: performed the experiment, analyzed the data, writing—original draft, writing. H-ZX, W-LL, C-JL and HLu: performed the experiment and writing—review and editing. HLi: funding acquisition. All authors reviewed the results and approved the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The 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.

## Publisher's note



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# Elevated sea temperature combined with dietary methionine levels affect feed intake and appetite-related neuropeptide expression in the brains of juvenile cobia (*Rachycentron canadum*)

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This study aimed to determine the impact of elevated temperature combined with different levels of dietary methionine concentrations on feed intake (FI) and brain expression of selected neuropeptides and one receptor involved in appetite control in juvenile cobia (approximately 3.7 g body weight). The genes studies were neuropeptide y, *npy;* agouti-related protein, *agrp*; cocaine- and amphetamine-regulated transcript, *cart;* cholecystokinin, *cck* and melanocortin 4 receptor; *mc4r*. The cobia were reared at typical sea water temperature in Vietnam (30 °C) and elevated temperature (34°C; selected as one of the predicted scenarios of climate change). The fish were fed diets with different levels of methionine: deficient (M9; 9.1 g/kg), sufficient (M12; 12.8 g/kg) and surplus (M16, 16.8 g/kg) for 6 weeks (triplicate tanks per treatment). Both dietary methionine concentration and temperature affected FI in cobia. Dietary methionine deficiency (i.e., M9) and elevated temperature reduced FI in cobia. Temperature significantly influenced the mRNA expression of *agrp*, *cart*, *cck* and *mc4r*. Expression of the orexigenic neuropeptide *npy* was consistently higher before the meal than after the meal for all diets and at both temperatures. At 30°C, prefeeding levels of *npy* correlated with both increased methionine levels and FI. The interaction between dietary methionine and temperature on the levels of brain *npy* expression was significant ($P<0.05$). There was higher brain expression of *agrp, cart* and *cck* in cobia at 34°C than in fish at 30°C, which was correlated with a lower FI. In conclusion, both feeding, temperature and/or dietary methionine levels affected the brain expression of *npy* and *agrp, cart, cck* and

*mc4r*. This suggests that these neuropeptides as well as the mc4r receptor are actively involved in adjusting feed intake to compensate for changing energetic demands, as well as metabolic adjustments due to the variable availability of methionine at elevated temperature.

KEYWORDS

cobia, appetite, feed intake, neuropeptide, gene expression, elevated temperature, dietary methionine

## 1 Introduction

Due to the ectothermic nature of fish, the environmental temperature is a key and main extrinsic factor that directly and significantly affects the rates of all physiological processes and metabolic pathways. Temperature strongly influences voluntary feed intake, feed utilization, and growth performance [see review by (Volkoff and Rønnestad, 2020)]. Based on the sixth assessment report (AR6) of climate change by IPCC (2021), under the worst scenario (SCP5-8.5), the highest increased level of net global temperature will be approximately 4°C by 2100. Such elevated temperatures will negatively affect most fish species (Glencross and Bermudes, 2011; Glencross and Bermudes, 2012; Sandersfeld et al., 2015; Pham et al., 2021), including cobia. Cobia (*Rachycentron canadum*) is a candidate species in aquaculture due to its high quality flesh and remarkable rapid growth (Chou et al., 2001). However, elevated temperatures reduce growth performance in the species (Sun and Chen, 2014; Nguyen et al., 2019), potentially representing a severe challenge to the large-scale commercial aquaculture of cobia worldwide and in Vietnam. A previous study in this species revealed that elevated temperature affects digestion with changes in the daily pattern of gastrointestinal pH, enzymatic proteolytic digestive activity and resulting increased feed transit time (Yúfera et al., 2019). However, it is still unknown to what extent cobia can adapt to high temperatures and whether dietary modifications may alleviate some of the potentially affected physiological systems controlling appetite and feed intake.

Methionine is an indispensable amino acid (IAA) in mammals and in fish. Besides being a precursor to protein synthesis, this IAA is also involved in several metabolic pathways, including fatty acid oxidation, phospholipid status, creatine synthesis, bile acid production and polyamine availability (Bender, 2012; Andersen et al., 2016; Espe et al., 2016). Supplementation of methionine in the diets influences the epigenetic modification of DNA methylation and gene expression and thus affects the growth and health of animals [reviewed by (Zhang, 2018)]. Methionine is a limiting AA in most plant ingredients, the dominant protein-rich source in aquafeeds, and is increasingly used in carnivorous species (Conceição et al., 2012). In cobia, the requirement of methionine is established at 11.9 g $kg^{-1}$ dry diet (26.4 g $kg^{-1}$ dietary protein) in the presence of 6.7 g $kg^{-1}$ cysteine (Zhou et al., 2006). Supplementation of crystalline DL-methionine in plant protein-based diets results in better growth and feed conversion ratio in cobia (Nguyen et al., 2019) as a consequence of the synchronization of the absorption rate between crystalline DL-methionine and protein-bound methionine (He et al., 2021).

Feed intake in fish, as in other vertebrates, is controlled by neuropeptides released in neural circuits in the brain that also receive inputs from afferent nerves, hormones and metabolic factors released in peripheral tissues (Rønnestad et al., 2016; Volkoff, 2016b; Soengas et al., 2018; Soengas, 2021). Among the appetite-controlling neuropeptides, neuropeptide Y (Npy) and Agouti-related peptide (Agrp) , are considered the most potent orexigenic (feeding stimulatory) factors (Nguyen et al., 2013; Rønnestad et al., 2016; Volkoff, 2016b; Soengas, 2021; Tolås et al., 2021). Intracerebroventricular injections of Npy in goldfish *Carassius auratus*, trout *Oncorhynchus mykiss* and channel catfish *Ictalurus punctatus* resulted in a dose-dependent increase in voluntary feed intake (López-Patiño et al., 1999; de Pedro et al., 2000; Narnaware et al., 2000; Silverstein and Plisetskaya, 2000; Aldegunde and Mancebo, 2006; Matsuda et al., 2012). A lower feed intake is correlated with reduced mRNA expression of *agrp* (Delgado et al., 2017; Soengas, 2021), whereas fasting leads to increased hypothalamic expression levels of *agrp* in goldfish (Cerdá-Reverter and Peter, 2003), zebrafish *Danio rerio* (Song et al., 2003) and Yafish *Schizothorax prenanti* (Wei et al., 2013), supporting the orexigenic role of Agrp in these species. In cobia, the presence and role of this neuropeptide in the brain is still not determined.

In contrast, cocaine- and amphetamine-regulated transcript, CART (Butler et al., 2017) and cholecystokinin (CCK) are believed to play anorexigenic (feeding inhibitory) roles in feeding control pathways in terrestrial animals and fish (Schwartz et al., 2000; Morton et al., 2014; Rønnestad et al., 2016; Volkoff et al., 2017). Increased expression levels of *cart* after feeding, suggesting a satiety-inducing effect, have been reported in several fish species, such as medaka *Oryzias latipes* (Murashita and Kurokawa, 2011), blind cavefish *Astyanax mexicanus* (Volkoff, 2016a), pacu *Piaractus mesopotamicus* (Volkoff et al., 2017) and Atlantic salmon *Salmo salar* (Murashita et al., 2009; Burt et al., 2013; Kousoulaki et al., 2013). CCK produced and secreted in the intestine has a key role in digestion in the vertebrates studied and is also believed to provide a key signal to the brain inducing satiety that terminates a meal. CCK is also produced in the brain, where a reduced feed intake is correlated with increased expression levels (summarized by (Volkoff, 2016b). While CCK previously have been described in cobia (Nguyen et al., 2013), no information is available on the structure and role of CART in cobia.

The melanocortin 4 receptor (MC4R) was characterized and reported to be involved in feeding behavior and the regulation of metabolism, homeostasis and growth in vertebrates, including fish (Volkoff, 2016b; Delgado et al., 2017; Rønnestad et al., 2017; Kalananthan et al., 2020a; Kalananthan et al., 2020b). This receptor is part of the melanocortin system (Anderson et al., 2016). In rainbow trout, *Oncorhynchus mykiss* (Schjolden et al., 2009), and goldfish (Cerdá-Reverter and Peter, 2003; Schjolden et al., 2009), there was a reduced feed intake after treatment with an Mcr agonist, while Mcr antagonist injection led to an increased feed intake (Cerdá-Reverter and Peter, 2003), supporting the anorexigenic effects of Mc4r in the studied species. However, no information is available on the structure and the role of Mcr4 in cobia.

Previous studies in mammalian species have shown that AAs participate in controlling feed intake via nutrient signaling pathways. This includes the olfactory and gustatory systems that affect feeding behavior and modulate the motivation to search and ingest food. Additionally, sensors in enteroendocrine cells releasing hormones such as CCK and postabsorptive signals such as nutrient sensing mechanisms in the liver, adipose tissue and brain are sensitive to AA levels (reviewed by (Fromentin et al., 2012). Recent research on how specific nutrients affect appetite and nutrient sensing pathways in fish has revealed more details on some of the involved mechanisms. For instance, intracerebroventricular (IC) injection of valine in rainbow trout resulted in increased feed intake, while IC injections of leucine led to reduced levels of *npy* and *agrp* (Conde-Sieira and Soengas, 2017; Delgado et al., 2017; Comesaña et al., 2018). Wang et al. (2021) reported that a dietary deficiency of methionine in largemouth bass, *Micropterus salmoides*, affected AA concentrations in plasma and activated nutrient signaling pathways at both cellular and systemic levels to regulate metabolism and control growth. In cobia, studies (Chi et al., 2020; He et al., 2021) have shown the involvement of methionine in peripheral signaling pathways that affect growth (Chi et al., 2020; He et al., 2021). However, the link between methionine and appetite control was not explored in any of these studies.

The present study aimed to target the role of the selected neuropeptides *npy, agrp, cart, cck* and receptor *mc4r* in appetite regulation pathway; and to explore to what extent the selected neuropeptides are related to the modulation of feed intake caused by dietary methionine concentrations (9.1, 12.8 and 16.8 g $kg^{-1}$, representing deficient, sufficient, and surplus levels of methionine, respectively) and temperatures (elevated 34°C vs. control 30°C). This study also aimed to determine potential interactions, if any between dietary methionine level and temperature on feed intake and brain expression of *npy, agrp*, *cart, cck* and *mc4r* in cobia.

# 2 Materials and methods

## 2.1 Experimental diet

Three experimental diets were prepared as described by (Nguyen et al., 2019), all isoenergetic (approximately 20.0 MJ/kg) with approximately 46,5% protein and 10,3% lipid and nutritionally balanced for all indispensable AAs except for methionine. Briefly, crystalline DL-methionine was added at different levels to make final diets that were deficient in methionine M9 (9.1 g $kg^{-1}$), sufficient in methionine M12 (12.8 g $kg^{-1}$) and surplus in methionine M16 (16.8 g $kg^{-1}$) for cobia juveniles, according to (Zhou et al., 2006; NRC, 2011; Nguyen et al., 2019). The dietary AA composition is presented in Table 1S (Supplemental data). All diets were produced at SPAROS Lda. (Olhão, Portugal).

## 2.2 Fish and experimental design

Cobia juveniles purchased from a local hatchery in Nha Trang, Vietnam, were stocked into two fiberglass tanks (5 $m^3$; 600 individuals per tank), assigned to elevated and control temperature fish groups, and acclimated for 1 week. During this time, fish were fed to satiation twice daily (at 8:00 and 16:00) with pelleted feed (INVE, Ltd. Ho Chi Minh City, Vietnam), and all uneaten feed was collected (Figure 1S). During the experiment, seawater temperature in the hatchery (indoor condition) varied between 27.0°C (nighttime) and 30.5°C (daytime). At the start of the trial, water temperature in the elevated temperature fish group tanks was gradually increased, with a rate of 1°C per day, until it reached 34°C by using thermal controllers (Chuan Kang Ltd., Taiwan). Thereafter the temperature for both fish groups was continuously maintained at 34°C and 30°C for remainder of the trial. Other water parameters were controlled and remained at 29±3.1 ppt salinity, pH of 7.8–8.3, 4.6±0.5 mg $L^{-1}$ oxygen and < 0.03 mg $L^{-1}$ total ammonia.

After this initial acclimation period, 180 fish from each temperature group (30 and 34°C) with uniform size (3.7±0.1 and 3.8±0.1 g body weight, respectively) were collected and distributed randomly into the experimental tanks at a density of 20 fish per tank. Eighteen experimental tanks (80×50×60 cm, 200 L) were connected to two recirculation subunits (nine tanks per unit with the same temperature) with continuous aeration. Output water from the rearing tanks of each unit was collected via perpendicular pipes (Ø 27 mm) in the middle of each tank and gathered in a reservoir tank (1.0 x 2.0 x 2.0 $m^3$) before it was pumped into a two-chamber organic filtered fiberglass tank (1.0 x 1.5 x 2.0 $m^3$) (for details see Nguyen et al., 2014). There seawater was passed through a sediment filter, sterilized and finally temperature adjusted before joining the recirculation system subunits. Thermal controllers (Chuan Kang Ltd., Taiwan) maintained the temperature at 30±0.1°C and 34±0.1° C in the two subunits. Other water parameters were maintained as in the acclimation period.

The trial started when fish were introduced to the experimental diet (feeding protocol as in the acclimation period) and lasted six weeks. At each temperature, groups of three tanks (triplicates) were randomly assigned to one of three diets: M9, M12 or M16 (as described in 2.1). Feed intake and growth performance were measured and calculated as described by Nguyen et al. (2019).

## 2.3 Sampling and sample preparation

Juvenile cobia were weighed individually and measured for length at the beginning and end of the experiment. The fish were anesthetized by MS-222 (Merck, Darmstadt, Germany; 0.4 mg $L^{-1}$)

prior to any handling. To target the role of the selected neuropeptide and receptor genes *npy, agrp, cart, cck* and *mc4r* in the appetite controlling pathways and determine the impact of dietary methionine and elevated temperature on expressions, cobia brain samples were collected just before feeding (prefeeding) and after feeding a meal (postfeeding). The prefeeding and postfeeding cobia from the same tank were sampled on different days to prevent the potential impact of handling stress on the meal effects on the expression of appetite genes. Two individuals from each tank were collected at the relevant sampling time.

The prefeeding unfed cobia were collected prior to the morning meal, from 08:00-09:00, while the postfeeding fed fish were collected 30 minutes after the meal was terminated. The fish were sampled by dip net and immediately anesthetized (MS-222; 0.4 mg $L^{-1}$), and then the whole brains were dissected and transferred into 4 mL cryo-vials prefilled with RNAlater (Thermo Fisher Scientific, MA, USA) on ice. The samples were kept overnight at 4°C in a refrigerator and then stored at −80 ± 3°C until they were transported (on dry ice) to the University of Bergen, Norway, where molecular cloning and quantitative PCR (Q-PCR) were performed.

## 2.4 Molecular cloning and Q-PCR

### 2.4.1 RNA isolation

Both *npy* and *cck* have previously been cloned in cobia (Nguyen et al., 2013), while the gene transcript sequences for *agrp*, *cart* and *mc4r* for this species are described in this study. We used the whole brain for cloning and establishment of Q-PCR assays for these genes. The tissues were homogenized using FastPrep FP120 (Savant Instruments, Holbrook, NY, USA), and the procedures followed the manufacturer's protocol. Total RNA was isolated using TRI Reagent (Sigma-Aldrich, MO, USA), and the RNA purity and concentration were checked in a NanoDrop ND-1000 (Thermo Fisher Scientific, MO, USA). The RNA integrity number (RIN) was assessed using the Agilent RNA 6000 Nano Assay protocol (Agilent Technologies).

### 2.4.2 cDNA synthesis

For each sample, 2 µg and 4 µg of total RNA templates were used to synthesize the first-strand cDNA for cloning and for qPCR, respectively, following the SuperScriptTM III Reverse Transcriptase protocol (Invitrogen, CA, USA) with oligo (dT)20 primers (Sigma-Aldrich, MO, USA). For each run, negative controls (no reverse transcriptase enzyme and no template controls) pooled RNA from all running samples were included.

### 2.4.3 Molecular cloning

Primers for the cloning of *agrp*, *cart* and *mc4r* were made based on sequence homology searches to similar species. The primers used are shown in Table 1. Nested PCR for cloning *agrp* was performed using the following thermal program: 98°C for 30 sec; 35 cycles at 98°C for 10 sec, 65°C for 30 sec, 72°C for 60 sec; and 72°C for 2 min using 50 ng of template. To clone *agrp*, Q5® High-Fidelity DNA Polymerase Enzyme (New England Biolabs, Ipswich, MA, USA) with enhancer was used. To clone *mc4r* and *cart* One*Taq*® DNA Polymerase enzyme (New England Biolabs, Ipswich, MA, USA) with GC buffer were used.

Nested PCR for cloning of *mc4r* was performed according to the following thermal program: 94°C for 2 min; 35 cycles at 94°C for 30 sec, 51°C for 60 sec, and 68°C for 30 sec; and 68°C for 5 min using 50 ng of template. Nested PCR for cloning of *cart* was performed using the thermal program 94°C for 2 min; 45 cycles at 94°C for 30 sec, 50°C for 60 sec, 68°C for 60 sec; followed by 68°C for 5 min using 50 ng of template. TOPO cloning (Invitrogen, Carlsbad, CA, USA) was performed according to the manufacturer's protocol, and sequencing was performed using the University of Bergen sequencing service. Q-PCR products were sequenced to verify the amplicon.

### 2.4.4 Quantitative PCR

Relative expression of gene transcripts was determined by Q-PCR using 1X iTaq™ SYBR® Green supermix (Bio-Rad), with 25 ng cDNA template (1:40) in 25 µl reactions following the manufacturer's protocol using 40 nM specific forward and reverse primers (Table 1). qPCRs were set up in multiple plates using the sample-maximization strategy (Bustin et al., 2010), with triplicates for each sample. Setups included no template and no reverse transcriptase control and between-plate positive controls. The thermal profile was 95°C for 3 min, followed by 40 cycles of 95°C for 30 sec, 58°C for 30 min, and 72°C for 30 sec, with a final denaturation step of 10 sec at 95°C. Among three housekeeping genes, beta-actin (*β-actin*; (Mohamed et al., 2007), elongation Factor 1alpha (*ef1a*; Gene Bank[1,2]) and ubiquitin protein ligase E3A [*ube3a*; (Betancur et al., 2013)], tested with cobia brain samples, *ef1a* was the most stable and thus was used as the reference gene for normalizing gene expression. Gene expression efficiencies for *ef1a*, *npy*, *agrp, cart, mc4r* and *cck* were calculated as 92.0, 98.1, 101.4, 97.4, 96.7 and 98.7%, respectively, using a dilution series for each assay. Mean normalized expressions (MNE) were calculated in the Microsoft Excel-based Application coded in Visual Basic for Applications, Q-Gene software package (Muller et al., 2002).

## 2.5 Statistical analyses

Statistical analysis was performed using SPSS for Windows (IBM® SPSS® Statistics version 25). Linear mixed models (in which Tanks is a random factor) were applied to test any differences and/or the interaction between feeding status (prefeeding vs. postfeeding), dietary methionine concentrations and temperature (as fixed factors) on FI, body growth and mRNA expression levels. Pairwise comparisons using Tukey's *post hoc* test were then applied to evaluate the differences between treatment means. Prior to performing these analyses, all data were checked for normal distribution (Kolmogorov-Smirnov test), homogeneity in variance (Levene's test) and heteroscedasticity (F test). Data on the

1 https://www.ncbi.nlm.nih.gov/nuccore/257062718

2 https://services.healthtech.dtu.dk/service.php?SignalP-5.0

TABLE 1 Primer sequences for cloning and Q-PCR. Primers for cloning indicated with c, primers for Q-PCR denoted with q. Amplicon length (bp) indicated for q-pcr assays.

| Gene | GeneBankNo | Primer | Primer sequences (5´-3´) | Amplicon length (bp) |
|---|---|---|---|---|
| *agrp* | OM962990 | *cAgRPF*<br>*cAgRPR*<br>*qAgRPF*<br>*qAgRPR* | ATGTTTGGCTCTGTGCTGCT<br>TCAGGTGCGTCTGGGAGG<br>CAGCAGTCATGCCTGGGTTA<br>GTTGAGAGCGGCAGTAGCA | 72 |
| *cart* | OM962989 | *cCARTF*<br>*cCARTR*<br>*qCARTF*<br>*qCARTR* | ATGTGGGCGCGAGCTG<br>TCATAAGCACTTCAGC<br>TCAAGTCCCCACGTGTGAT<br>CGCACATCTTCCCAATCCGA | 70 |
| *mc4r* | OM962988 | *cMC4RF*<br>*cMC4RR*<br>*qMC4RF*<br>*qMC4RR* | ATGAACACCACAGAATATCATGGA<br>TCACACACACAAGAGAGCGT<br>ATCACCAGCATCTGGACGTG<br>AGCACCAGCATGGTGAAGAA | 113 |
| *ef1a* | FJ653664 | *qEF1AF*<br>*qEF1AR* | GCTAGTGGAACCACACTGCT<br>TTCAGGACGCCAGTCTCAAC | 152 |

Primers for NPY and CCK for cloning and qPCR; see Nguyen et al., (2013).

mean normalized expression (MNE) of *npy* were found to be inhomogeneous in variance and heteroscedasticity, and a natural logarithm was applied for MNE (x1000) before applying the tests. Correlation of MNE among genes was performed by applying the Pearson test. Graphs were generated using the ggplot2 package version 3.3.5 (Wickham, 2016) in R 3.1.3 and Rstudio 1.1.463.

## 2.6 Multiple alignments, structural analysis, and phylogeny

Predicted protein sequences for cobia Agrp, Cart and Mc4r were aligned using Genious software. Signal peptide cleavage analysis for Agrp was performed using SignalP Ver. 5.0[3,4] program. Putative signal peptides were predicted using PrediSi[5,6], and proteolytic cleavage sites were predicted using Neuropred[7,8]. Transmembrane regions, including extracellular and intracellular loops, as well as identifying cysteines involved in disulfide bonds, were retrieved by blasting cobia Mc4r in UniProt[9,10] and performing transmembrane topology prediction using the deep TMHMM[11] sequence predictor transmembrane domains.

All peptide sequences were aligned using MUSCLE with the default parameters (UPGMA clustering method, Gap opening penalty −2.90, Gap extension 0.0) from MEGAX (Hall, 2013). Phylogenetic analysis was performed using the predicted mature peptide sequences. Based on best-fit substitution model analysis in MEGAX, a phylogenetic tree was constructed using maximum likelihood (Huggett et al., 2005) with a Jones-Taylor-Thornton (JTT) model (Jones et al., 1992; Kumar et al., 2018) with a fixed gamma distribution (+G) parameter with five rate categories and 500 bootstrap replicates. The trees were rooted to the human, *Homo sapiens*, sequences. Cobia Cart were aligned to all known paralogs and rooted to the human, elephant shark, *Callorhinchus milii*, coelacanth, *Latimeria chalumnae, and* Cart sequences before being subjected to phylogenetic analysis using only Cart2 transcript variants. In the initial analysis, we included fish species as old teleost Mexican tetra, *Astyanax mexicanus*, Atlantic herring, *Clupea harengus* and salmonids, rainbow trout and Atlantic salmon (that went through a 4 round of whole genome duplication; 4R WGD), fish species before the whole teleost specific genome duplication event (Ts WGD) (spotted gar, *Lepisosteus oculatus*, and elephant shark), and species that diverged before 4R WGD (northern pike, *Esox lucius*) and those that went to the very recent 4R WGD (goldfish) and zebrafish which did not go to 4R WGD (Ravi and Venkatesh, 2018).

# 3 Results

## 3.1 Feed intake and growth

The results on feed intake and growth in cobia were reported and discussed by (Nguyen et al., 2019) and are only summarized here (Figure 1). Generally, cobia were very active during feeding in all groups, and initiated feeding as soon as the first pellet was dropped into the tank. The fish were apparently satiated and typically stopped ingesting pellets after 5-7 min of feeding. The

3 https://services.healthtech.dtu.dk/service.php?SignalP-5.0

4 http://stagbeetle.animal.uiuc.edu/cgi-bin/neuropred.py

5 http://www.predisi.de/home.html

6 https://dtu.biolib.com/DeepTMHMM/

7 http://stagbeetle.animal.uiuc.edu/cgi-bin/neuropred.py

8 http://stagbeetle.animal.uiuc.edu/cgi-bin/neuropred.py

9 https://www.uniprot.org/blast/

10 https://zfin.org/ZDB-GENE-150212-1#summary

11 https://dtu.biolib.com/DeepTMHMM/

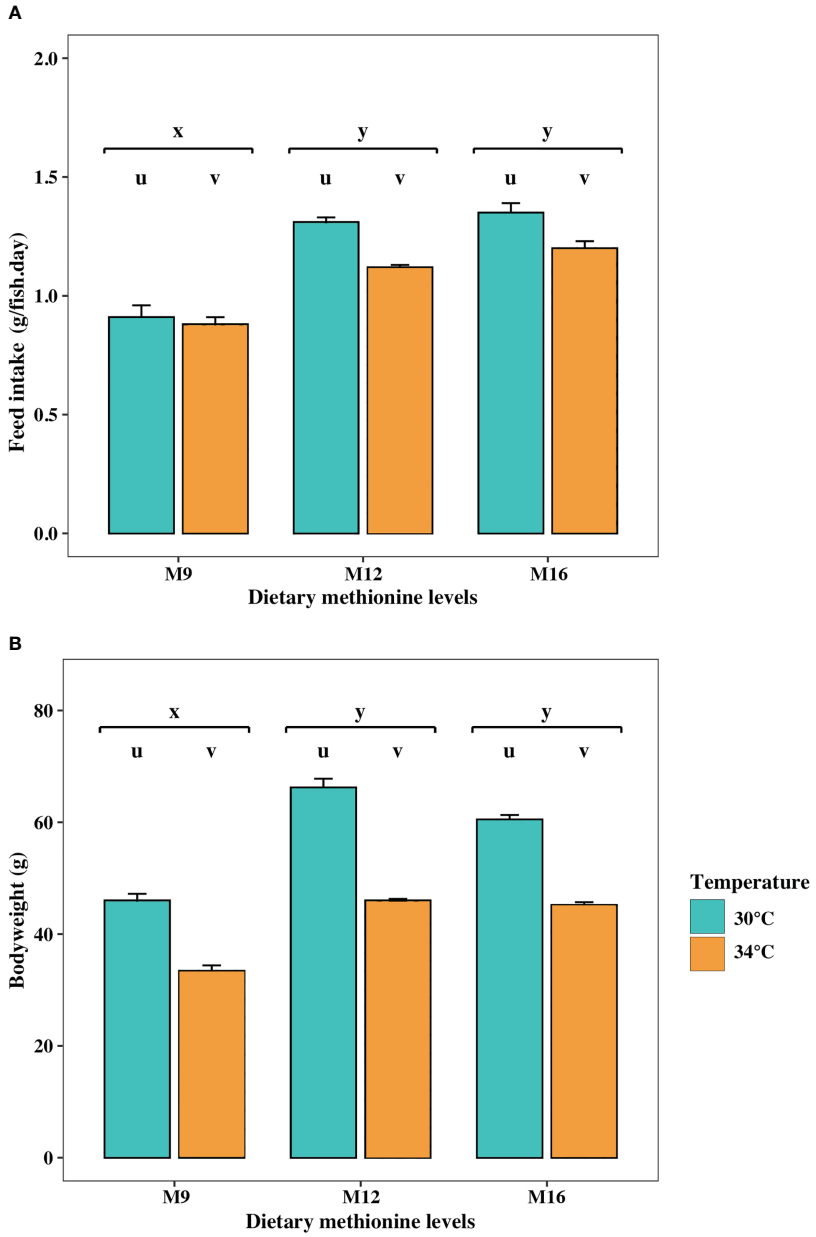


FIGURE 1
**(A)** Feed intake (FI) and **(B)** final body weight in cobia fed different methionine diets [9.1 $gkg^{-1}$ (M9, deficient), 12.8 $gkg^{-1}$ (M12, sufficient) and 16.8 $gkg^{-1}$ (M16, surplus)] and at two temperatures (30°C and 34°C). Bars present mean with standard errors, using tanks as the statistical unit (N=3). Different letters on top of boxes indicate significant difference ($p<0.05$) between temperatures (u, v) and among methionine levels (x, y).

levels of feed intake at M12 and M16, calculated over the six-week duration of the trial, did not differ, while growth was better at M12. Both Groups M12 and M16 had a higher feed intake and better growth compared to the M9 fish. Higher feed intake at 30°C resulted in higher growth in this treatment compared to the fish reared at 34°C. While interaction effects between dietary levels of methionine and temperature were present in growth, these two factors acted independently on feed intake.

## 3.2 Molecular cloning and phylogeny

The cobia *npy* and *cck* nucleotide sequences were reported in our previous study [GenBank accession no, KC284716 and KC284715 (Nguyen et al., 2013)]. For the cobia *agrp*, *cart* and *mc4r*, we obtained the ORF sequences from the PCR: Agrp (GenBank accession no OM962990), Cart (GenBank accession no OM962989) and Mc4r (GenBank accession no OM962988).

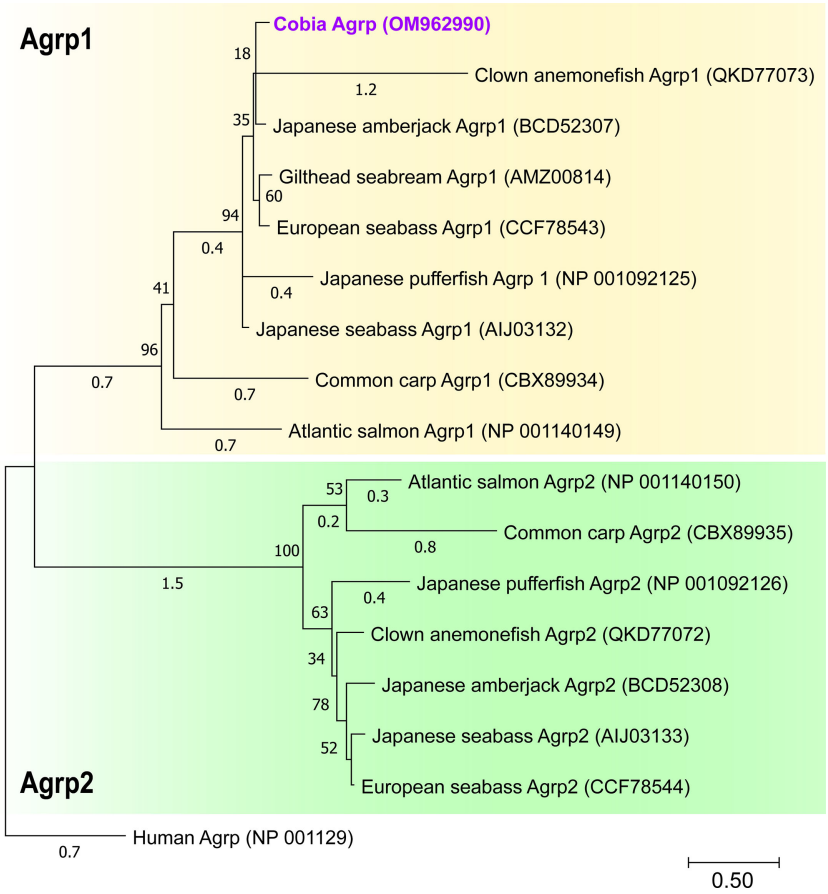


FIGURE 2
Phylogenetic analysis of the predicted cobia *Agrp* mature peptide sequence. Alignments were made using the submitted GenBank protein sequences in MEGA-X software. All peptide sequences were aligned using MUSCLE with the default parameters. The phylogenetic tree was constructed using Maximum Likelihood (ML) with a Jones-Taylor-Thornton (JTT) model with fixed Gamma distribution (+G) parameter with five rate categories and 500 bootstrap replicates. The tree was rooted to the human *Agrp* sequence. GenBank Accession Nos are as follows: cobia (*Agrp*, OM962990), Clown anemonefish (*Agrp1*, QKD77073), Japanese amberjack (*Agrp1*, BCD52307), Gilthead seabream (*Agrp1*, AMZ00814) European seabass (*Agrp1*, CCF78543), Japanese pufferfish (*Agrp1*, NP_001092125), Japanese seabass (*Agrp1*, AIJ03132), common carp (*Agrp1*, CBX89934), Atlantic salmon (*Agrp1*, NP_001140149), Atlantic salmon (*Agrp2*, NP_001140150), common carp (*Agrp2*, CBX89935), Japanese pufferfish (*Agrp2*, NP_001092126), Japanese seabass (*Agrp2*, AIJ03133), clown anemonefish (*Agrp2*, QKD77072), Japanese amberjack (*Agrp2*, BCD52308) and European seabass (*Agrp2*, CCF78544) and outgroup Human (*Agrp*, NP_001129).

The cobia Agrp (OM962990) is 426 bp in length and encodes sequences of 141 AAs (Figure 2S). The molecular characterization of cobia Agrp is shown in Figure 2S (alignment of AA sequences) and Figure 2 (phylogenetic analysis of mature peptide sequence). The signal peptide cleavage site is between pos. 20 and 21: SSS-LV (P:0.2927) (Figure 2S). The cobia Agrp sequence clusters well within the teleost Agrp1 clade and differs significantly from Agrp2 identified in teleosts. The cobia Agrp showed 50.7% amino acid identity to human AGRP (Figure 2).

The cobia Cart paralog identified in this study (OM962989) is 312 bp in length and encodes a sequence of 103 AAs (Figure 3S). The molecular characterization based on AA alignment of cobia Cart clusters within the Cart2a clade (Figure 3). Cobia Cart2a contains an 18-amino acid residue putative signal peptide[12]. The deduced cobia Cart2a protein has six cysteine residues for a putative disulfide bond that is conserved in Cart proteins, and the processing signals (KR and KK) are conserved in cobia Cart (Figure 3S). Cobia Cart2a structural analysis indicated a cleavage site between positions 23 and 24 of TEA-ER[13] (results not shown). Cobia Cart2a showed 65.3% amino acid identity to human CARTs (Figure 3).

The cobia Mc4r (OM962988) is 975 bp in length and encodes sequences of 324 AAs (Figure 4S). The molecular characterization of cobia Mc4r is provided in Figure 4S (AA alignment) and Figure 4 as a phylogenetic analysis. By blasting the cobia Mc4r sequence in UniProt and the deep TMHMM sequence predictor[14], the transmembrane domains and extracellular and intracellular loops for cobia Mc4r were identified. The cysteines involved in the disulfide bonds in human MC4R are conserved in cobia Mc4r (results not shown). The cobia Mc4r sequence clusters well within

12 http://www.predisi.de/index.html

13 http://stagbeetle.animal.uiuc.edu/cgi-bin/neuropred.py

14 https://dtu.biolib.com/DeepTMHMM/

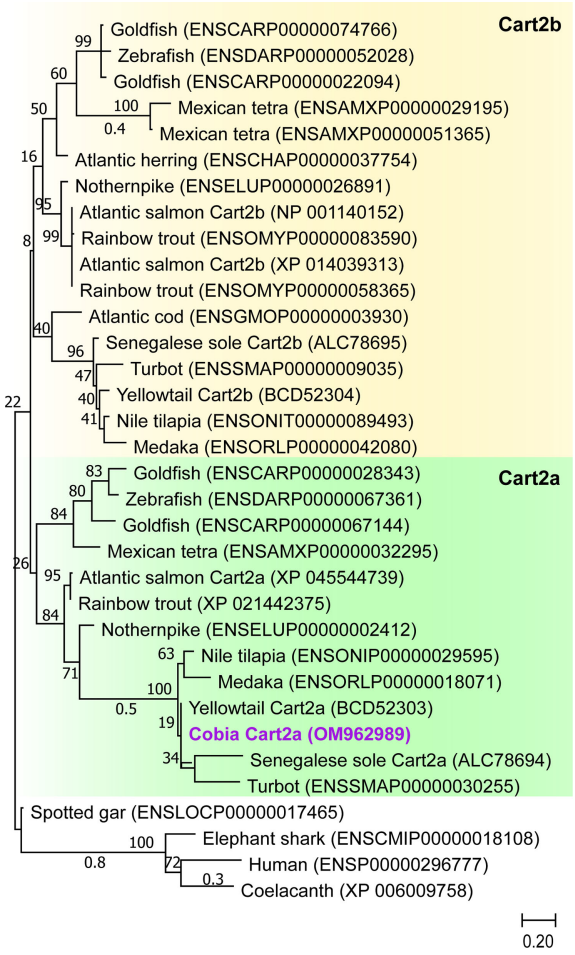


FIGURE 3
Phylogenetic analysis of the predicted mature peptide sequence of cobia *Cart*. Alignments were made using the submitted GenBank peptide sequences in MEGA-X software. All peptide sequences were aligned using MUSCLE with the default parameters. The phylogenetic tree was constructed using Maximum Likelihood (ML) with a Jones-Taylor-Thornton (JTT) model with fixed Gamma distribution (+G) parameter with five rate categories and 500 bootstrap replicates. The tree was rooted to the human, elephant shark and coelacanth *Cart* sequences. GenBank Accession Nos and ENSEMBL are as follows: Cobia (*Cart2a*, OM962989), Atlantic salmon (*Cart2a*, XP_045544739) Atlantic salmon (*Cart2b*, NP_001140152 and XP_014039313), Yellowtail (*Cart2a*, BCD52303), Yellowtail (*Cart2b*, BCD52304), human (*Cart*, ENSP00000296777), Coelacanth_(*Cart*, XP_006009758), Spotted gar_(*Cart*, ENSLOCP00000017465), Goldfish (*Cart2*, ENSCARP00000074766, ENSCARP00000067144, ENSCARP00000028343 and ENSCARP00000022094), zebrafish (*Cart2*, ENSDARP00000052028 and ENSDARP00000067361), Nothernpike (*Cart2*, ENSELUP00000002412 and ENSELUP00000026891), Rainbow_trout_(*Cart2*, ENSOMYP00000058365, ENSOMYP00000083590 and XP_021442375), Atlantic_herring (*Cart*, _ENSCHAP00000037754), Atlantic_cod (*Cart*, _ENSGMOP00000003930), Nile_tilapia_(*Cart2*, ENSONIT00000089493 and ENSONIP00000029595) and Senegalese_sole_(*Cart2a*, ALC78694), Senegalese_sole_(*Cart2b*, ALC78695), Turbot (*Cart2*, ENSSMAP00000030255 and ENSSMAP00000009035), Medaka (*Cart2*, ENSORLP00000042080 and ENSORLP00000018071), Mexican tetra (*Cart2*, ENSAMXP00000029195, ENSAMXP00000051365 and ENSAMXP00000032295) and Elephant_shark (*Cart*, ENSCMIP00000018108).

the teleost Mc4r clade (Figure 4). The cobia Mc4r showed 68.1% amino acid identity to human Mc4R (Figures 4, 4S).

## 3.3 Expression of brain neuropeptides

### 3.3.1 Quality control of results in Q-PCR analysis

The mean RIN value of the extracted RNA brain samples was 8.8 (SD=0.6). All samples had RIN values above eight and could therefore be used for downstream applications (Fleige and Pfaffl, 2006). To evaluate the potential for a minor degradation of RNA in the brain samples during sampling or storage, regression analysis was performed, indicating a weak correlation between RIN values and Cq values for *efa1*, *npy*, *agrp*, *cart* and *cck* (results not shown). This indicates that minor degradation did not affect the gene expression results.

### 3.3.2 *npy* expression- effects of temperature, methionine levels and a meal

Temperature did not significantly affect the expression of *npy* (P>0.005). However, dietary methionine levels affected the cobia's brain expression of *npy*. Cobia fed the M16 diet showed a higher level of *npy* than those fish fed the M9 diet; while cobia fed the M12 diet showed comparable brain expression of *npy* to the fish fed the other diets. In addition, levels of brain *npy* in the cobia analyzed around the first meal in the morning were higher before (pre) feeding than after (post-) feeding in all experimental treatments ($P = 0.001$, Figure 5A).

There was an interaction between dietary methionine and temperature on *npy* expression of cobia's brain (P=0.014) that led to higher level of *npy* in cobia fed M16 diet than those fish fed the M9 diet at control temperature (30°C), but not at elevated temperature (34°C).

### 3.3.3 *agrp* expression- effects of temperature, methionine levels and a meal

Expression of *agrp* in the brain of cobia was affected by both temperature ($P < 0.001$) and intake of a meal (pre vs. post feeding of the first meal in the morning) ($P = 0.009$), but not methionine levels (Figure 5B). *agrp* mRNA levels were higher in the fish reared at elevated temperature (34°C) than the ones reared at control temperature (30°C). Also, the prefeeding cobia showed higher *agrp* than the postfeeding fish. There were no significant combined effects among feeding, dietary methionine, and rearing temperature on the expression levels of this gene.

### 3.3.4 *cart*, *cck* and *mc4r* expression- effects of temperature, methionine levels and a meal

Temperature affected expression of these neuropeptides and the receptor. The elevated temperature group had higher expression levels of cart (P< 0.001), cck (P=0.002) and mc4r (P< 0.0001) in the cobia brain (Figures 5C–E). There were no significant differences in

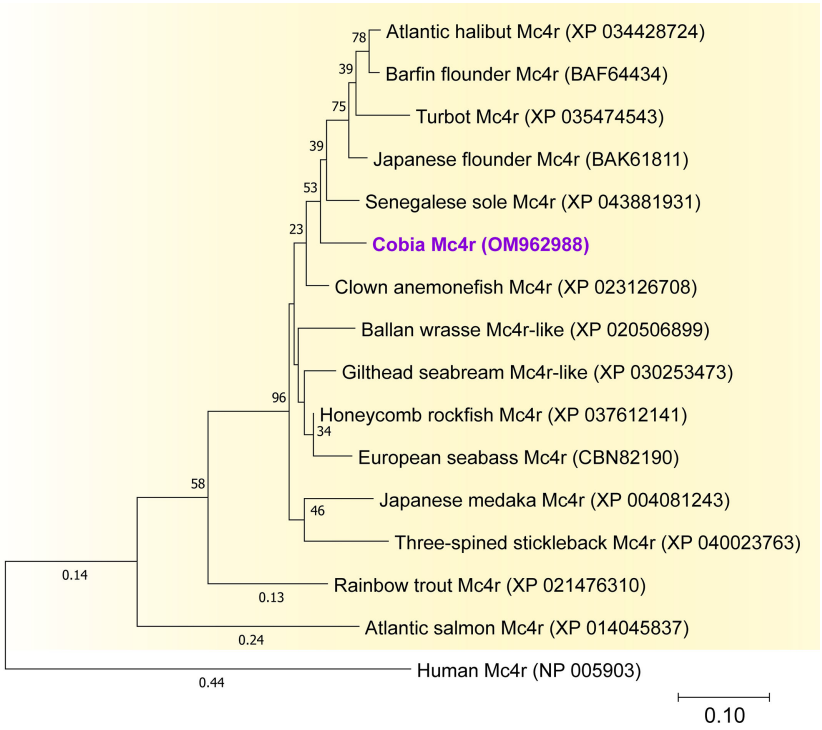


FIGURE 4
Phylogenetic analysis of the mature peptide sequence of cobia *Mc4r*. Alignments were made using the submitted GenBank amino acid sequence in MEGA-X software. All peptide sequences were aligned using MUSCLE with the default parameters. The phylogenetic three was constructed using Maximum Likelihood (ML) with a Jones-Taylor-Thornton (JTT) model with fixed Gamma distribution (+G) parameter with five rate categories and 500 bootstrap replicates. The tree was rooted to the human *Mc4r* sequence. GenBank Accession Nos are as follows: Japanese medaka (*Mc4r*, XP_004081243), Cobia (*Mc4r*, OM962988), Three-spined stickleback (*Mc4r*, XP_040023763), Senegalese sole (*Mc4r*, XP_043881931), Japanese flounder (*Mc4r*, BAK61811), Gilthead seabream (*Mc4r-like*, XP_030253473), Atlantic halibut (*Mc4r*, XP_034428724), Clown anemonefish (*Mc4r*, XP_023126708), Honeycomb rockfish (*Mc4r*, XP_037612141), Barfin flounder (*Mc4r*, BAF64434), Ballan wrasse (*Mc4r-like*, XP_020506899), Turbot (*Mc4r*, XP_035474543), European seabass (*Mc4r*, CBN82190), Rainbow trout (*Mc4r*, XP_021476310), Atlantic salmon (*Mc4r*, XP_014045837) and outgroup Human (*Mc4r*, NP_005903).

brain expressions of cart, cck and mc4r amongst the cobia fed different dietary methionine levels, nor between the prefeeding and postfeeding fish (P>0.05).

### 3.3.5 Correlation analysis of gene expression

The correlation analysis of gene expression (Figure 5S) showed values ranging from -0.02 (cart and npy) to 0.83 (agrp and cart).

In summary, dietary methionine affected the brain expression levels of *npy*, while elevated temperature caused significantly upregulated expression of *agrp*, *cart*, *cck* and *mc4r*. Expression levels of both *npy* and *agrp* were higher in cobia before the meal (prefeeding) compared to after the meal had been terminated (postfeeding). There was a combined effect of dietary methionine and rearing temperature on the expression levels of *npy*.

## 4 Discussion

### 4.1 Effects of dietary methionine levels and temperatures on feed intake and growth performance

In the present study, cobia fed the M9 diet had the lowest feed intake, indicating that a dietary deficiency of methionine reduced appetite in the fish (Figure 1A). As expected, the fish that was fed diet deficient in methionine (M9 diet) also grew less (Figure 1B) and had a poorer FCR, compared to those fish fed either the M12 or the M16 diets. The dietary deficiency of one indispensable AA will lead to an increased oxidation of other indispensable and dispensable AAs present in the diets, thereby reducing growth in the cobia [see a more detailed discussion in (Nguyen et al., 2019)]. These results are in line with previous studies on cobia related to methionine availability, growth and feed utilization (Zhou et al., 2006; Van Nguyen et al., 2014; Wang et al., 2016; Chi et al., 2020), as well as in other fish species (Walton et al., 1982; Murthy and Varghese, 1998; Elmada et al., 2016; Gao et al., 2019; Wang et al., 2021). The finding that dietary deficiency of methionine is also involved in the loss of appetite and reduction of FI, resulting in reduced growth and feed efficiency in cobia, has also been observed in European sea bass (Thebault et al., 1985), grouper *Epinephelus coioides* (Luo et al., 2005), turbot *Scophthalmus maximus* (Peres and Oliva-Teles, 2005), gilthead sea bream *Sparus aurata* (Sánchez-Lozano et al., 2011), large yellow croaker *Pseudosciaena crocea* (Mai et al., 2006; Li et al., 2021) and golden pompano, *Trachinotus ovatus* (Niu et al., 2013). Although, feed intake in cobia fed M12 and M16 diets did not differ, growth was better in the fish fed the M12 diet (Nguyen et al., 2019).

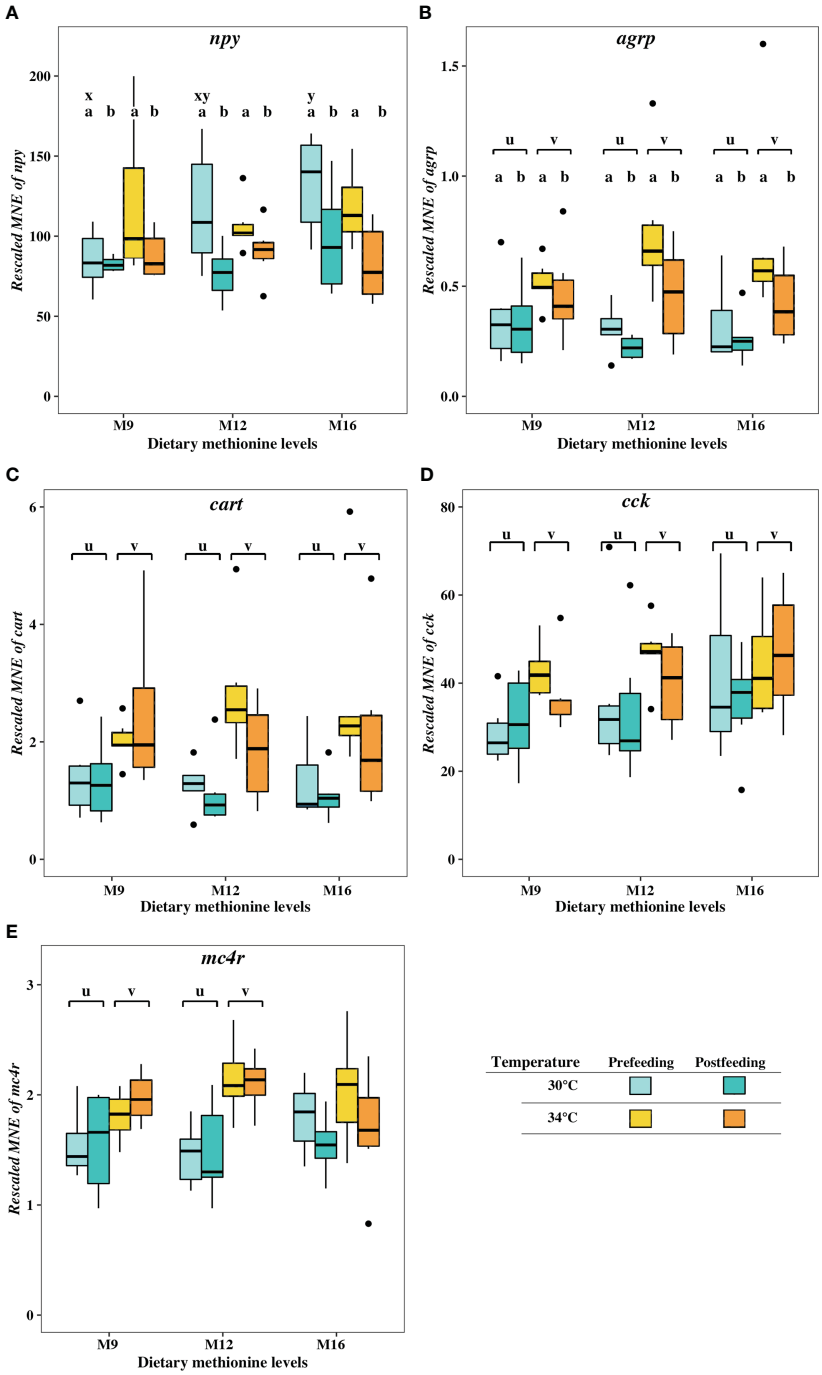


FIGURE 5
Relative mRNA expression of neuropeptides **(A)** *npy*, **(B)** *agrp*, **(C)** *cart*, **(D)** *cck* and **(E)** the melanocortin receptor *mc4r* in the brain of cobia fed three dietary methionine levels (9.1 $gkg^{-1}$ (M9), 12.8 $gkg^{-1}$ (M12) and 16.8 $gkg^{-1}$ (M16)) (x-axis) at two temperatures (30°C and 34°C and; before (Pre-feeding) and after (Post-feeding) a meal. Values shown are for triplicate tanks with six fish per group (n = 3). Y-axis shows rescaled mean normalized expression ($MNEx10^3$). Data is shown as boxplots with median (black line within the box), 25th and 75th percentiles (boundaries closest and farthest to zero) and 10th and 90th percentiles (whiskers above and below the box). Points outside the 10th and 90th percentiles are outliers. Different letters on top of boxes indicate the significant difference ($p<0.05$) between temperatures (u, v), two feeding states (a, b) and among methionine levels (x, y).

The elevated temperature had a negative effect on fish performance. Both feed intake and growth were lower in cobia juveniles reared at 34°C than at 30°C (Figure 1). With increasing temperature, the rates for all metabolic and physiological processes in an ectothermic fish will increase up to a certain level (Hevrøy et al., 2011; Volkoff and Rønnestad, 2020). Performance is maximal in an optimal temperature range but will then start to decline and proceed toward zero at the upper critical temperature (Volkoff and Rønnestad, 2020). The data in this study indicate that 34°C is beyond the optimum temperature for cobia but still below the

critical thermal maximum (Sun and Chen, 2014). Sun and Chen (2014) reported that cobia shows the maximal growth rate at 31°C, which shows that this species can effectively ingest and allocate sufficient energy to growth at this temperature.

The growth result from the current study suggests that there were higher costs for routine metabolism, and combined with a lower feed intake, a smaller fraction of the energy budget could be allocated to growth in cobia kept at 34°C compared to the fish at 30°C. This result compares well with that reported for other fish species exposed to temperatures above the optimum range (Glencross and Bermudes, 2011; Glencross and Bermudes, 2012; Sandersfeld et al., 2015; Pham et al., 2022). Although fish will reduce their feed intake when the temperature increases over their optimal range, there is little information on the underlying signaling pathways and the involvement of central appetite-controlling neuropeptides.

## 4.2 *npy* and *agrp* expression- effects of temperature, methionine levels and a meal

Feeding resulted in a decline in the expression levels of both brain *npy* and *agrp* in the cobia after the meal at both temperatures and for all three diets (Figures 5A, B). This supports the notion that Npy and Agrp peptides are involved in the control of appetite and feeding and that both act as orexigenic factors in fish (Volkoff, 2016b; Delgado et al., 2017; Rønnestad et al., 2017), including cobia (Nguyen et al., 2013). The described time course fits an orexigenic peptide that stimulates feeding behavior and intake of feed with a higher activity before than after the meal, when the fish is satiated. The present study thus supports other notions that orexigenic activity is also reflected in the mRNA expression of specific peptides.

Previous studies have suggested that Npy is the most potent orexigenic player involved in central control of appetite and feed intake of fish. Several studies have reported that the expression levels of *npy* decline after a meal; Atlantic cod, *Gadus morhua* (Kehoe and Volkoff, 2007), zebrafish (Tian et al., 2015), and grass carp, *Ctenopharyngodon idella* (Zhou et al., 2013). In goldfish, brain *npy* mRNA levels increase before and are reduced after a meal, again supporting this notion (Narnaware et al., 2000). Feed deprivation and fasting also result in elevated levels of *npy*. This suggests, as expected, that hunger signals increase in fasted fish, including coho salmon (*Oncorhynchus kisutch*), chinook salmon (*Oncorhynchus tshawytscha*), goldfish, and winter skate (*Leucoraja ocellata*) (Silverstein et al., 1998; Narnaware and Peter, 2001; MacDonald and Volkoff, 2009). Following the same argument, refeeding goldfish resulted in a reduction in *npy* to normal levels, which also supports an orexigenic role of Npy (Narnaware and Peter, 2001).

An orexigenic effect of Npy is also reflected in the fact that cobia fed the M9 diet had a lower *npy* expression level and a lower feed intake, and therefore also a lower growth rate compared to the fish fed either the M12 or M16 diets. Thus, the imbalanced dietary AA profile did not induce a compensatory orexigenic drive resulting in an increased feed intake. In contrast, our data show high expression levels of *npy* before the onset of feeding in the M12 and M16 groups (Figure 5A). It should be noted that the prefeeding expression levels were analyzed 30 min before the meal was administered. The underlying mechanisms for having an orexigenic drive or a sense of hunger before the meal are partly expected to be due to intrinsic signals from reduced filling of the gastrointestinal tract and directly or indirectly from the nutrient and energy status of the body. However, given that the fish were fed according to a fixed feeding schedule, this could also involve a conditioned anticipatory response, with the fish expecting a meal at this time. Such conditional anticipation, assessed as altered behavior when food is offered on a periodic basis, has been reported for several species, including Atlantic salmon (Fernö et al., 1995; Folkedal et al., 2012).

The mechanism for why insufficient dietary methionine led to a lower orexigenic action compared to sufficient and surplus methionine may involve a range of nutrient sensing pathways; either methionine acts as an attractant and increasing palatability in cobia or other intrinsic signaling pathways, possibly directly in the brain or in the liver acting via secondary endocrines, which both may induce an orexigenic action in *npy* expression. The underlying mechanism of this action in the brain may involve methionine sensing pathways that affect mTOR (target of rapamycin), ERK [extracellular signal-regulated kinase, and mitochondrial metabolism of AAs, which results in the activation of ROS (reactive oxygen species) and elevated levels of ATP/ADP from the mitochondria of the cell, as proposed for branched-chain AA by (Conde-Sieira and Soengas, 2017)]. In mammals, it has been shown that hepatic FGF21 is the key mediator of the physiological and behavioral effects related to metabolism and energy balance caused by methionine-restricted diets (Forney et al., 2018).

Interestingly, Forney et al. (2017) showed that restriction of dietary methionine induced hyperphagia in mice. They proposed that a methionine-sensing system is linked to a compensatory hyperphagic mechanism, but only when there is sufficient methionine in the diet to prevent weight loss through a compensatory increase in feed intake, thus pointing to a concentration-dependent and essential AA-specific effect of the AA composition of the diet. Further studies are required to clarify to what extent similar links between the sensing of specific AAs and appetite control are found in cobia as well as in other ectotherms.

With regard to temperature effects, it is also interesting to note that the *npy* behavior related to the intake of a meal differed between the two temperatures. The correlation between *npy* expression and the methionine level was not observed at the elevated temperature. This could be due to a possible stress response caused by temperatures beyond the optimum. A link between NPY and stress has been reported for mammals (Reichmann and Holzer, 2016), but whether there is a relationship between Npy and stress in cobia remains to be explored. On the other hand, our data suggest that the appetite signaling pathways via Npy action in cobia are impacted mainly by the presence of food (prefeeding vs. postfeeding) and food quality (methionine deficiency) rather than environmental temperature.

Agrp has also been reported to be involved in the regulation of energy homeostasis and stimulation of food intake in teleosts (Sohn, 2015; Takeuchi, 2016). Fasting leads to increased expression levels of *agrp* in the hypothalamus of goldfish (Cerdá-Reverter and Peter,

2003), zebrafish (Song et al., 2003), and Ya fish *Schizothorax prenanti* (Wei et al., 2013), indicating an orexigenic role of this neuropeptide in appetite control in fish (Volkoff, 2016a). To date, we have only identified one paralog of *agrp* in the cobia brain (Figures 2, 2S), compared to two reported paralogs of *agrp* (*agrp* 1 & 2) that play different roles in controlling appetite in mammals and some fish species (e.g., clownfish (*Amphiprion ocellaris)*, (Pham et al., 2021), Atlantic salmon (Murashita et al., 2009; Murashita and Kurokawa, 2011; Kalananthan et al., 2020b; Lai et al., 2021) and zebrafish (Shainer et al., 2017)). Our analysis shows that the presently reported *agrp* in cobia aligns well with other teleost *agrp1* paralogs (Figure 2). Additionally, our results on cobia *agrp* are in line with those reports for *agrp* in Arctic charr, *Salvelinus alpinus* (Striberny et al., 2015), and for *agrp1* in transgenic coho salmon, (Kim et al., 2015) where *agrp* levels decline after feeding. act as an orexigenic neuropeptide. In contrast, the other *agrp* paralog, *agrp2*, showed an increased expression level after feeding in clownfish (Pham et al., 2021). In Atlantic salmon, the two *agrp* paralogs have different distributions in the brain and respond differently to feeding status. (Kalananthan et al., 2020b) showed that *agrp1* had the highest expression in the hypothalamus and was affected by feeding status (fasting), while *agrp2* had higher expression levels in other brain regions (i.e., in the *saccus vasculosus*) and did not respond to fasting. The reported *agrp2* is also known as *asip2* and may therefore serve different roles than appetite control, i.e., in zebrafish, it has been described to provide negative regulation of cortisol secretion and pigmentation[15].

In the current study, dietary methionine did not affect the brain expression of *agrp in cobia*, indicating that this neuropeptide did not respond to any nutrient-sensing mechanisms for methionine. In addition, elevated temperature resulted in higher expression levels of *agrp*, even though the FI in this treatment was lower than that in the control. These results may indicate that *agrp* responds to signals related to long-term energy status and demands rather than appetite in response to elevated temperature.

The upregulation of *agrp* at the mRNA level in cobia at prefeeding and at elevated temperature suggests that this neuropeptide not only acts as an orexigenic factor but is also involved in the regulation of energy homeostasis in fish, as reported in mammals (see (Sohn, 2015) for a review). To cope with elevated temperature (an unfavorable thermal condition), a higher abundance of *agrp* could be expected in cobia for the fish to control its body homeostasis as an adaptive strategy. Thus, upregulation of the *agrp* gene may partly contribute to maintaining appetite to compensate for the higher metabolic cost due to elevated temperature. However, for cobia kept at 34°C, any potential compensatory mechanisms to increase FI to keep up with the metabolic demands were inhibited by anorexigenic mechanisms, resulting in a lower FI (Figure 1). In zebrafish, acute stress induced a rapid increase in total body cortisol and increased central expression levels of *npy* and *agrp* as a coordinated response to counteract the inhibitory effects of stress on feed intake (Cortes et al., 2018). In the present study, fish were acclimated to elevated temperatures for a long time, and any stress-related response to the increased temperature was not acute but long-term. In addition, we only observed increased expression levels of *agrp* and not *npy*. It is thus possible that in cobia kept at elevated and unfavorable temperatures, two orexigenic neuropeptides respond differently based on their individual involvement in signaling pathways that include both stress and energy homeostasis. More studies are encouraged to explore and elucidate such mechanisms.

In short, the results from the present study indicate that *npy* and *agrp* act as orexigenic neuropeptides in pathways of appetite and feeding control in cobia, but with different roles and final effects on the feed intake and impacts of elevated temperature. Dietary methionine significantly affected the expression levels of *npy*, while elevated temperature led to increased *agrp* expression in cobia.

## 4.3 *cart* and *cck* expression- effects of temperature, methionine levels and a meal

In this study, we were able to identify and characterize a single Cart transcript gene in the cobia transcriptome, while there are multiple paralogs identified in some teleosts, such as ten in Atlantic salmon (Kalananthan et al., 2021) seven in Senegal sole, *Solea senegalensis* (Bonacic et al., 2015), and six in medaka (Murashita and Kurokawa, 2011). The cobia *cart* aligns with the *cart2a* gene family reported for other teleost species (Figure 3) and responds to feeding status. The transcriptome of cobia larvae and pooled tissue has recently been described (Ebeneezar et al., 2023), but as of now, no characterization of brain cobia is available. Thus, a more detailed exploration of the cobia genome and brain regional analysis expression profiles is required to conclude if cobia has other cart paralogs involved in appetite control. It is known that the early evolution of vertebrates was accompanied by two rounds of WGD and that thereafter WGD is only known in ray-finned fish and amphibian lineages. Similar to salmonids, suckers have also experienced recent tetraploidization events (Ravi and Venkatesh, 2018).

Both *cart* and *cck* mRNA levels were higher at higher temperatures. Since both are expected to give anorexigenic effects, this may be the direct link to lower appetite and feed intake at 34°C in cobia. The higher temperature might have caused a stress effect that stimulated increased activity of these neuropeptides. Although not directly comparable, a study of the effects of short-term stress on appetite-related genes in zebrafish indicated that while the expression of orexigenic genes was not affected by handling, the anorexigenic genes analyzed, including *cart*, showed increased expression after stress, thereby suggesting a direct involvement of Cart in the anorexigenic effect of stress (Cortes et al., 2018). Our data support this notion in cobia.

However, the brain expression of *cart* and *cck* did not change during a meal, nor were there any differences among the three dietary methionine concentrations (Figures 5C, D ). Thus, there was no clear direct role of Cart and Cck levels in appetite control in the brain related to meal or methionine level control of cobia. CART and CCK have been reported to act as satiety factors that suppress feed intake and terminate ingestion in many vertebrates, including fish (Crawley and Corwin, 1994; Himick and Peter, 1994; Peyon et al., 1999; Gélineau and Boujard, 2001; Volkoff et al., 2003;

15 https://zfin.org/ZDB-GENE-150212-1#summary

Matsuda et al., 2012; Morton et al., 2014; Conde-Sieira and Soengas, 2017). For instance, intracerebroventricular (ICV) administration of Cart resulted in reduced feed intake in several species of fish, such as goldfish (Volkoff and Peter, 2000) and channel catfish (Kobayashi et al., 2008). In addition, feed deprivation led to decreased expression levels of *cart* in Atlantic cod (Kehoe and Volkoff, 2007), goldfish (Volkoff and Peter, 2001), and Atlantic salmon (Murashita et al., 2009), while refeeding resulted in increased *cart* abundance in channel catfish (Peterson et al., 2012), goldfish (Volkoff and Peter, 2001), and dourado, *Salminus brasiliensis* (Volkoff et al., 2017). Insulin treatment increased *cart* in rainbow trout (Libran-Perez et al., 2015) and catfish (Subhedar et al., 2011). Both central and peripheral injections of Cck reduced feed intake in rainbow trout (Gélineau and Boujard, 2001; Jönsson et al., 2006), coho salmon (White et al., 2016), goldfish (Himick and Peter, 1994; Volkoff et al., 2003; Kang et al., 2010), catfish (Silverstein and Plisetskaya, 2000), sea bass (Rubio et al., 2008), and winter flounder, *Pseudopleuronectes americanus* (MacDonald and Volkoff, 2009). Higher *cck* mRNA levels after ingesting feed have been reported in goldfish brain (Reimers et al.), yellowtail, *Seriola lalandi* pyloric cecum (Reimers et al.) and Atlantic salmon brain (Peyon et al., 1999; Murashita et al., 2007; Valen et al., 2011). Meanwhile, the administration of CCK antagonists resulted in an increase in feed intake in rainbow trout (Gélineau and Boujard, 2001).

Whereas the highest expression levels of *cck* in the goldfish brain were observed in the hypothalamus (Peyon et al., 1999), this information is not available for juvenile cobia. Therefore, regional differences in changes in cobia brain *cck* levels were not clarified. CCK is found not only in the brain but also in the digestive tract, where it serves as a key regulator of the digestive process, regulates gallbladder release, stimulates the release of pancreatic enzymes, enhances motility, and regulates stomach evacuation (Jensen et al., 2001; Murashita et al., 2006). CCK acts locally in the gut and via enteric and vagal signaling, where it acts on appetite and behavioral control of feed intake. Ingestion of nutrients induces the release of CCK, which relays first to the hindbrain and ultimately to the hypothalamus and brain reward regions (Shechter and Schwartz, 2018). Expression levels of both *cck-l* and *cck-n* from the gastrointestinal tract increased within 1.5 hours after a meal (Rønnestad et al., 2010). Thus, to obtain the complete involvement of CCK in the gut-brain axis during a meal, both organs must be assessed in parallel.

Any change in *cck* expression after a meal may take some time, and some of the dynamic changes in *cck* expression could have been missed in our experiment due to the sampling strategy. In goldfish, the expression levels of *cck* in the brain increased 2 h postprandial (Peyon et al., 1999). In Atlantic salmon, there were different patterns of changes in the expression of different isoforms of brain *cck* after feeding; levels of *cck-l* were elevated at 12 h postprandial, while there were no changes in the expression of *cck-n* (Valen et al., 2011).

The gene for pro-opiomelanocortin (POMC) is a precursor for well-known neuropeptide appetite suppressors in the hypothalamus that is also coexpressed with CART in mammals (Schwartz et al., 2000; Soengas et al., 2018). However, *pomc* was not successfully cloned in cobia, and primers for qPCR were not specific for the detection of levels in *pomc* cobia brain samples. Thus, we do not currently have a good understanding of the integration of these two important neuropeptides related to metabolic and endocrine signals involved in the control of feed intake.

However, higher levels of *cart* and *cck* in cobia at 34°C, concomitant with lower feed intake compared to those fish at 30°C (Figure 1A), indicate that these two neuropeptides may act as anorexigenic factors that reduce feed intake in cobia. Prolonged exposure to unfavorable elevated temperatures likely led to the induction of anorectic signals, resulting in reduced appetite and thus feed intake and thus having negative effects on growth performance in cobia. The apparent paradoxical reduction in feed intake at elevated temperatures is possibly linked to a decrease in aerobic scope that results from higher temperatures (Nilsson et al., 2009). Based on data on clownfish, (Pham et al., 2022) proposed that a reduced meal size limits the standard dynamic action (SDA; increased oxygen consumption after a meal) beyond the aerobic scope to prevent impacts that reduce performance.

## 4.4 *mc4r* expression- effects of temperature, methionine levels and a meal

The integration between nutrient sensing and neuroendocrine signaling in regulating appetite is very complex and not yet fully understood in fish (Soengas, 2021). MC4R is known to be involved in the regulation of feed intake and homeostasis/energy expenditure and growth in vertebrates, including fish (Cerdá-Reverter and Peter, 2003; Tao, 2010; Rønnestad et al., 2017). MC4R serves as an integrating receptor in second-order neurons, where its activity is inhibited when stimulated by orexigenic neuropeptides and its activity is increased by competitive anorexigenic neuropeptides (Reimers et al., 1993; Cone, 2006). In cobia, we found only one form of Mc4r that is clustered together within the teleost clade, suggesting an important role in integrating neuronal activity on appetite control and feed intake in cobia. In many teleost species, Mc4r is characterized by more than one paralog (e.g., four Mc4r in Atlantic salmon (Kalananthan et al., 2020a), three in goldfish (Reimers et al.) and one in common carp (Reimers et al.)). To what extent these paralogs play different roles remains to be explored.

In the present trial, *mc4r* expression was higher at the higher temperature, possibly suggesting a link with the general lower feed intake in cobia at 34°C (Figure 5E). In rats, administration of an MC4R antagonist (SHU9119) increased feeding and induced obesity, suggesting the anorexigenic effects of MC4R on feed intake and homeostasis of the animals (Hagan et al., 1999; Hwa et al., 2001). Additionally, long-term ICV infusions with MC4R antagonists such as HS014 and HS024 in rats result in increased food intake and body weight (Kask et al., 1998; Skuladottir et al., 1999). Mc4r has been characterized in several fish species (Rønnestad et al., 2017; Liu et al., 2019; Kalananthan et al., 2020a), even though the impact of expression levels and the role of this receptor in appetite regulation is still unclear (Kobayashi et al., 2008; Sanchez et al., 2009; Pham et al., 2021). For instance, studies in rainbow trout (Schjolden et al., 2009) and goldfish (Cerdá-Reverter and Peter, 2003; Schjolden et al., 2009) showed reduced feed intake after treatment with an Mcr agonist, and Mcr

antagonist injection led to increased feed intake in these species (Cerdá-Reverter and Peter, 2003), supporting the anorexigenic effects of Mc4r. On the other hand, progressive fasting did not affect the expression of *mc4r* in the hypothalamus of barfin flounder *Verasper moseri* (Kobayashi et al., 2008) and European sea bass (Sanchez et al., 2009). In the current study, there were no significant differences between prefeeding and postfeeding cobia among dietary methionine and temperature. It is possible that *mc4r* expression levels vary in different brain regions, with high expression in the preoptic and arcuate nuclei of the brain (Tao, 2010; Liu et al., 2019; Wang et al., 2020). In addition, the role of Mc4rs varies from tissue to tissue. MC4R expressed in the paraventricular nucleus of the hypothalamus is correlated with anorexigenic effects, i.e., inhibition of feed intake, while MC4R expressed by sympathetic neurons within the intermediolateral column of the spinal cord is believed to increase energy expenditure (Sohn, 2015). However, higher levels of *mc4r* at 34°C correlated with lower FI and poorer growth in cobia, suggesting that Mc4r is involved in the anorexigenic stimulation that suppresses feed intake in this species.

Although we cannot demonstrate the link between dietary methionine levels and *mc4r* expression, the response of *mc4r* expression at high temperature differed in fish fed different levels of methionine. In addition, in humans, the protein profile in restricted diets influenced appetite and craving differently in individuals with different gene variants of MC4R (Huang et al., 2017). In this regard, more attention is needed to clarify the role of dietary methionine levels on appetite in fish, including the impact of Mc4r.

To date, some reports in mammals have shown that the limitation of essential AAs alters the expression level of anorexigenic neuropeptides in the hypothalamus [reviewed by (Forney et al., 2018)]. However, our results show that in cobia, the main response was observed in orexigenic *npy*. The signaling pathways in which restricted dietary methionine affects appetite in mammals seem to involve peripheral organs (e.g., hepatic FGF21) (Forney et al., 2018; Chi et al., 2020), but the pathways in the hypothalamus are still unclear.

## 5 Conclusion

Elevated temperature (34°C) and dietary methionine deficiency both reduced feed intake in cobia, resulting in retarded growth. Feeding cobia a dietary methionine concentration above the requirement did not promote growth performance, neither in the control nor at the higher temperature. Dietary methionine affected the expression levels of *npy*. Meanwhile, elevated temperature significantly affected and upregulated the expression of *agrp*, *cart*, *cck* and *mc4r*. There was a combined effect of dietary methionine and rearing temperature on cobia's brain expression levels of *npy*. In addition, expression levels of both *npy* and *agrp* were higher before compared to after the meal. At 30°C, prefeeding levels of *npy* were correlated with both increased methionine levels and feed intake. Taken together, these results support an orexigenic role of Npy and Agrp, as well as the anorexigenic role, and possibly stress-related effects caused by temperature, of Cart and Cck in the signaling pathway of appetite and feeding behavior in cobia. In summary, both feeding, temperature and/or dietary methionine levels affected the brain expression of *npy, agrp, cart, cck* suggesting that these neuropeptides and the receptor *mc4r*, are actively involved in adjusting feed intake to compensate for changing energetic demands as well as metabolic adjustments due to variable availability of an essential AA at elevated temperature.

## Data availability statement

The gene datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: GenBank, OM962988-OM962990. The remainder of the data supporting the conclusions of this article will be made available by the authors upon request, without undue reservation.

## Ethics statement

The animal study was reviewed and approved by Center for Experiments and Practices at Nha Trang University, Nha Trang, Vietnam, chaired by Dr. Nguyen Van Hoa.

## Author contributions

MN, LP, A-EJ, ME, LC, MY, SE, ML, and IR conceived and designed the study. MN and ML conducted the experiment. MN, LP, A-EJ, ME, LC, MY, SE, ML, and IR contributed to the sampling. MN and A-EJ performed the primer design, preparatory lab work and qPCR. A-EJ performed the phylogenetic analysis. MN and LP performed the statistical analysis. LP and MN drafted the manuscript. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

Author LC was employed by SPAROS Lda.

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.

## Publisher's note



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2023.1183967/full#supplementary-material

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# Replacement of fish oil by alternative n-3 LC-PUFA rich lipid sources in diets for European sea bass (*Dicentrarchus labrax*)

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**Introduction:** In order to ensure lipid quality of cultured fish and an environmentally sustainable production, new alternative sources of EPA and DHA are needed to replace traditional lipid sources, such as fish oil.

**Methods:** Different combinations of alternative marine lipid sources rich in n-3 LC-PUFA available in the market were herein evaluated to replace traditional fish oil (sardine oil) in diets for European sea bass (*Dicentrarchus labrax*). A commercial-type diet (CTRL), containing 1.6% of EPA + DHA, 5% sardine oil and 8% rapeseed oil was used as a negative control. Another diet (SARDINE) formulated with 8.5% sardine oil, 4.5% rapeseed oil and 2.5% EPA + DHA was used as the positive control. Three experimental diets were formulated to completely replace sardine oil with alternative sources, targeting approximately the same EPA + DHA level as the positive control: the SALMON diet contained 9.9% salmon by-product oil mixed with 3.1% of an algal oil rich in EPA and DHA, while the ALGARAPE and the ALGASOY diets included 4.4% of the algal oil and 8.6% of either rapeseed or soybean oil, respectively. A sixth diet (ALGABLEND) was formulated to partially replace sardine oil with salmon by-product oil and rapeseed oil, balanced with 2% of algae biomass. The experimental diets were hand-fed to 118 g fish for 54 days.

**Results:** All diets were well-accepted by fish and no significant differences were found in feed efficiency, growth performance, somatic indexes or whole body composition among treatments. At the end of the trial, regardless the dietary EPA + DHA level, all fillets contained more than 250 mg of EPA + DHA per 100 g fresh weight, meeting EFSA recommendations for cardiovascular risk prevention for European adults (> 250 mg day $^{-1}$).

**Discussion:** Overall, this study demonstrated that combining expensive sources of n-3 LC PUFA (Veramaris® or Algaessence Feed™ with low-priced sustainable oils (salmon by-products oil or vegetable oils) allows fortifying European sea bass flesh with EPA and DHA, without major textural changes. This approach is a successful strategy for mitigating the negative effects associated with the high inclusion of vegetable oils. However, the retention of n-3 LC-PUFA in muscle was not significantly increased, suggesting that there is a maximum dietary threshold beyond which β-oxidation might be promoted, and hence there is no advantage in increasing the dietary level of these fatty acids in European sea bass diets.

KEYWORDS

**algal oil, EPA and DHA, fish flesh quality, fish oil replacement, omega-3 fatty acids, sustainable aquaculture**

## Introduction

Fish is a rich source of high-quality protein and micronutrients, such as vitamins A, B12 and D, as well as minerals like iodine and selenium. It is also one of the few sources of omega-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA) for human consumption (Xu et al., 2020). Omega-3 LC-PUFA, particularly eicosapentaenoic acid (C20:5n-3, EPA) and docosahexaenoic acid (C22:6n-3, DHA), play an important role in neural system development and help reduce the risk of chronic pathologies, such as cardiovascular diseases, diabetes and obesity in humans (Li et al., 2020). Dietary recommendations for daily intake of EPA and DHA to prevent the risk of cardiovascular accidents in European adults are between 250 and 500 mg per day, according to the European Food Safety Authority (EFSA, 2012).

Aquaculture contributes to nearly half of global fish consumption (FAO, 2022), being a major source of EPA and DHA for the burgeoning human population. However, most marine fish have limited ability to convert short-chain ($C_{18}$) polyunsaturated fatty acids (PUFA) into LC-PUFA ($C_{20-22}$) (Monroig et al., 2018). As a result, fish farming is dependent on the dietary supply of n-3 LC-PUFA, which is traditionally obtained from fish oil (FO) and fish meal (FM) (Tocher et al., 2019). The dependence on small pelagic stocks to support aquaculture growth compromises environmental sustainability and adds increasing pressure on fishery resources, which risk being overexploited if not well managed (Naylor et al., 2021; FAO, 2022). Therefore, finding alternative sources of EPA and DHA for aquafeeds is imperative to ensure an adequate, sustainable and secure supply of raw-materials for future formulations.

The partial or total FO replacement by vegetable oils (VO) in fish diets has been thoroughly evaluated (Turchini et al., 2010; Torrecillas et al., 2017). Many studies have shown that it is feasible to partially replace FO by VO in diets for both freshwater and marine fish species without affecting feed utilization and growth (Turchini et al., 2009; Dikel et al., 2013; Pereira et al., 2019). However, others studies have reported negative effects on fish health and fillet nutritional value due to the low levels of n-3 LC-PUFA and the imbalance of n-3 PUFA to n-6 PUFA (Glencross, 2009; Nasopoulou and Zabetakis, 2012). In European sea bass (*Dicentrarchus labrax*), soybean (rich in linoleic acid C18:2n-6 (LA); 57% of total fatty acids) and rapeseed oil (rich in oleic acid C18:1n-9 (OA); 54% of total fatty acids), two highly available alternative lipid sources, have been used up to a 60% FO replacement without compromising growth (Izquierdo et al., 2003), but decreasing n-3 LC-PUFA levels in the fillet of commercial-sized fish (Izquierdo et al., 2005; Mourente and Bell, 2006). Such negative effects on final product quality may limit the benefits of fish consumption. While considerable efforts have been carried out to mitigate the negative impacts of replacing FO by VO in aquafeeds (Mourente and Bell, 2006; Turchini et al., 2010), some studies have identified a range of alternative EPA and DHA sources with the potential to improve aquafeeds (Tocher, 2015; Tocher et al., 2019; Santigosa et al., 2020; Santigosa et al., 2021; Kousoulaki et al., 2022).

The pressure to use local resources and adopt a circular economy strategy can increase the supply of marine FO and strongly contribute to a reduction of its carbon footprint (Naylor et al., 2009; Klinger and Naylor, 2012; Campos et al., 2020). In fact, recycling activities such as producing FO from fish waste or by-products of the processing industry are projected to ramp up in the coming years (FAO, 2022). According to FAO (2022), the global production of Atlantic salmon (*Salmo salar*) in 2020 was 2.71 million tons, accounting for 32.6% of finfish in marine and coastal aquaculture. Slaughter-sized Atlantic salmon contains about 45% of its lipids in the fillet whereas the other 55% are present in the inedible portions (Ytrestøyl et al., 2011). These by-products can be used to generate added-value products such as salmon oil and could, hence, be a valuable source of n-3 LC-PUFA for aquafeeds (Ytrestøyl et al., 2011; Deepika et al., 2014).

Although the use of existing n-3 LC-PUFA sources should be considered (Tocher, 2015), it has been recently defended that recycling cannot be the only solution to ensure the supply of EPA and DHA (Tocher et al., 2019). Algal biomass and algal oils have

just emerged as an alternative to FO and VO with promising results in several fish species (Miller et al., 2007; Kiron et al., 2012; Santigosa et al., 2020; Santigosa et al., 2021). Ferreira et al. (2021) have characterized the nutritional value of four commercially available micro- and macroalgae (*Nannochloropsis* sp., *Chlorella* sp., *Gracilaria* sp. and *Ulva* sp.) and a blend of the four algae species (Algaessence Feed™), reporting approximately 6% of lipids (as % dry matter, DM) in *Nannochloropsis* sp. and *Chlorella* sp., over 19 mg $g^{-1}$ of EPA in *Nannochloropsis* sp. and approx. 5 mg $g^{-1}$ of EPA in Algaessence Feed™, but none of the algae contained DHA. Other algae species (i.e. *Schizochytrium* and *Crypthecodinium* sp.) rich in DHA (30-40% of total FA) are available in the market (Adarme-Vega et al., 2012), and selected strains have been used for the production of valuable alga oils. Ferreira et al. (2022) observed an efficient accumulation of EPA+DHA in the muscle of gilthead seabream *(Sparus aurata)* fed a diet that included a blend of microalgae (including DHA-rich *Schizochytrium* sp.) to replace 33% FM. The use of a DHA and EPA rich oil obtained from *Schizochytrium* in FO-free diets has shown good growth and an efficient deposition of EPA and DHA in the muscle of gilthead seabream (at a 3.5% inclusion level, Santigosa et al., 2021) and rainbow trout *(Oncorhynchus mykiss)* (at a 5% inclusion level, Santigosa et al., 2020). Despite the encouraging results, the use of such algal oils to fully replace FO and restock the high levels of n-3 LC-PUFA of a FO-free diet is not yet economically viable (Naylor et al., 2021). Therefore, the use of multiple cost-effective lipid sources to avoid possible negative impacts of single ingredients, and maximize each ingredient's contribution to the aquafeed nutritional balance has been envisaged (Chauton et al., 2015; Zhang et al., 2019).

In this context, this study aimed to identify the best combination of alternative lipid sources rich in LC-PUFA and currently available in the market, to replace sardine oil in diets for European sea bass, but maintaining growth performance, nutrient utilization, and flesh quality for human consumption.

# Materials and methods

## Ingredients and experimental diets

Commercially available salmon by-product oil (SmO; Sopropêche, France), algae oil (AO; Veramaris®, Evonik, NE, USA) and Algaessence Feed™ (AF; ALGAplus Lda., Portugal), a blend of macro and microalgae species (*Gracilaria* sp., *Nanochloropsis* sp. and *Shizochitrium* sp.) were selected based on their n-3 LC-PUFA richness and economic feasibility to replace sardine oil (SdO), ensuring high EPA and DHA levels. The fatty acid profile of lipid sources is presented in Table 1 and described in the next section. Based on the nutritional requirements of European sea bass (NRC, 2011), six isonitrogenous (51% DM), isolipidic (18% DM) and isoenergetic (23 kJ $g^{-1}$ DM) diets were formulated with moderate levels of marine-protein ingredients (15% FM and 3.5% hydrolyzed fish protein), but differing in the oil sources (Table 2). A commercially-based diet was used as a negative control (CTRL): it provided 1.6% of EPA+DHA and included 5% SdO and 8% rapeseed oil (RO). A positive control diet (SARDINE), with 8.5% SdO and 4.5% RO, providing 2.5% of EPA+DHA was also tested. This diet implied a 6% increase in price compared to the CTRL. Three other diets were formulated to totally replace sardine oil by the selected alternative sources, targeting approximately the same EPA+DHA levels as the positive control. In the SALMON diet, 9.9% SmO and 3.1% AO were used, whereas in ALGARAPE and ALGASOY diets 4.4% AO and 8.6% of either RO or soybean oil (SyO) were included. These diets totally replaced sardine oil with a 16-17% increase in price compared to the CTRL. The last diet ALGABLEND was formulated to partially replace the SdO included in the positive control by 7.9% SdO and 4.7% RO balanced with 2% of AF biomass. This diet also targeted the same EPA+DHA level as in the positive control and explored the functionality of the algae biomass, but the drawback was a 41% price increase.

All diets were supplemented with mono-calcium phosphate, L-lysine and DL-methionine, and were extruded and produced by SPAROS Lda. (Olhão, Portugal). Proximate composition and fatty acid profile of the experimental diets are presented in Tables 2 and 3, respectively.

## Lipid sources and diets fatty acid profile

The FA profile of all selected lipid sources is presented in Table 1. Major differences were found in total SFA and MUFA: while SdO, AO and AF displayed higher percentages of SFA (>28%), RO, SmO and SyO had higher levels of MUFA, which resulted from a high percentage of oleic acid (C18:1n-9) ranging from 25.4 to 60.3% in these oils. The sum of PUFA also varied between lipid sources, ranging from 27.4 to 63.8%. AO had the highest PUFA sum, followed by SyO and SdO. Within PUFA, major differences were found in the level of both LA and α-linolenic acid (C18:3n-3, ALA), which were more than 10 times higher in SmO, RO and SyO than in other lipid sources. More importantly, SdO and AO were very rich sources of EPA (>20% and >17% of total FA, respectively) which was absent in RO and SyO. The DHA level was particularly high in AO (>40% of total FA), followed by AF (>30% of total FA), and was only present at 4% of total FA in SmO, and absent in RO and SyO. These differences were reflected in the sum of EPA and DHA (highest in AO, followed by AF and SdO) and DHA/EPA ratio, which was highest in AF. The n-3/n-6 ratio of the tested ingredients was highest in AO, followed by AF and SdO (Table 1). Consequently, all the diets had very different fatty acid profiles (Table 3). The CTRL diet had the lowest percentage of SFA (18% of total FA) and PUFA levels (36% of total FA), but the highest MUFA (44% of total FA); in CTRL, 10% of total FA was EPA+DHA (corresponding to 1.6 g of EPA+DHA per 100 g DM). All diets were richer in PUFA than the CTRL diet, and as expected, their levels of EPA+DHA (15.6 - 16.7% total FA, corresponding to 2.6 - 2.8 g 100 $g^{-1}$ DM) were close to the SARDINE diet (15.4% of total FA, corresponding to 2.5 g 100 $g^{-1}$ DM). A 3-4% dietary inclusion of AO in SALMON, ALGARAPE and ALGASOY almost doubled the DHA concentration in these diets. This increase was reflected on DHA/EPA ratio, which was highest in ALGASOY followed by ALGARAPE and SALMON. However, n-3/n-6 ratios

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TABLE 1 Fatty acid profile of lipid sources.

| | SdO | SmO | RO | SyO | AO | AF |
|---|---|---|---|---|---|---|
| Fatty acids (% total fatty acids) | | | | | | |
| C14:0 | 6.7 | 2.2 | 0.04 | 0.11 | 2.62 | 31.3 |
| C16:0 (PA) | 16.7 | 9.6 | 4.2 | 10.1 | 28.7 | 15.7 |
| C18:0 | 3.2 | 2.6 | 1.4 | 3.02 | 1.2 | 0.4 |
| **Σ SFA**[1] | 28.1 | 15.1 | 6.4 | 14.0 | 34.2 | 48.6 |
| C16:1 n-7 | 10.2 | 2.64 | 0.2 | 0.1 | 0.03 | 3.2 |
| C18:1n-9 (OA) | 7.9 | 38.8 | 60.3 | 25.4 | – | 0.7 |
| C18:1 n-7 | 3.4 | 2.9 | 2.9 | 1.6 | 0.03 | 0.3 |
| C20:1 n-9 | 1.3 | 3.2 | 1.3 | 0.3 | 0.01 | – |
| **Σ MUFA**[2] | 24.0 | 50.4 | 65.1 | 27.4 | 0.08 | 4.4 |
| C18:2 n-6 (LA) | 1.0 | 14.1 | 19.1 | 52.1 | 0.01 | 0.5 |
| C18:3 n-3 (ALA) | 0.5 | 6.1 | 8.1 | 5.4 | 0.05 | 0.1 |
| C18:4 n-3 | 2.5 | 0.7 | – | – | 0.3 | 0.2 |
| C20:4 n-6 | 1.2 | 0.2 | – | – | 2.2 | 1.9 |
| C20:5 n-3 (EPA) | 20.7 | 3.1 | – | – | 17.4 | 2.7 |
| C22:5 n-3 | 1.9 | 1.2 | 0.01 | – | 1.8 | – |
| C22:6 n-3 (DHA) | 8.0 | 3.6 | – | – | 41.0 | 32.1 |
| EPA+DHA | 28.7 | 6.7 | – | – | 58.4 | 34.8 |
| DHA/EPA | 0.4 | 1.2 | – | – | 2.4 | 11.8 |
| **Σ n-3**[3] | 34.5 | 16.0 | 8.3 | 5.6 | 61.4 | 35.5 |
| **Σ n-6**[4] | 2.7 | 15.5 | 19.2 | 52.2 | 2.4 | 2.7 |
| Σ n-3/Σ n-6 | 12.8 | 1.0 | 0.4 | 0.1 | 25.2 | 13.3 |
| **Σ PUFA**[5] | 42.6 | 32.1 | 27.4 | 57.8 | 63.8 | 38.2 |
| Fatty acids (g 100g$^{-1}$ DM) | | | | | | |
| C20:5 n-3 (EPA) | 19.3 | 2.9 | – | – | 16.2 | 0.7 |
| C22:6 n-3 (DHA) | 7.4 | 3.4 | – | – | 38.2 | 8.4 |
| EPA+DHA | 26.7 | 6.3 | – | – | 54.4 | 9.1 |

SdO, Sardine oil; SmO, Salmon oil; RO, Rapeseed oil; SyO, Soybean oil; AO, Algae oil; AF, Algaessence Feed™; PA, palmitic acid; OA, oleic acid; LA, linoleic acid; ALA, α-linolenic acid, EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; DM, dry matter.

[1] Σ SFA, sum of saturated fatty acids, includes C10:0, C12:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C22:0;

[2] Σ MUFA, sum of monounsaturated fatty acids, includes: C14:1, C16:1, C17:1, C18:1n-7, C18:1n-9, C20:1n-9, C22:1n-9, C22:1n-11, C24:1n-9;

[3] Σ n-3, sum of n-3 PUFA, includes:C18:3n-3, C18:4n-3, C20:3n-3, C20:4n-3, C20:5n-3, C22:5n-3, C22:6n-3;

[4] Σ n-6, sum of n-6 PUFA, includes: C18:2n-6, C18:3n-6, C20:2n-6, C20:3n-6, C20:4n-6;

[5] Σ PUFA, sum of polyunsaturated fatty acids, includes C16:2n-4, C16:3n-4, C16:4n-1, C18:2n-6, C18:3n-6, C18:3n-3, C18:4n-3, C20:2n-6, C20:3n-6, C20:4n-6, C20:3n-3, C20:4n-3, C20:5n-3, C22:5n-3, C22:6n-3.

were still below 1 for ALGARAPE and ALGASOY, and above 1 for SARDINE, SALMON and ALGAEBLEND (Table 3).

## Growth trial and fish sampling

The growth trial was conducted in Riasearch Lda. (Murtosa, Portugal), with European sea bass juveniles obtained from the commercial fish farm ALGAPlus Lda. (Ílhavo, Portugal). Fish were individually weighed and 12 homogeneous groups of 17 fish (118.6 ± 15.2 g) were established and distributed by 350 L fiberglass tanks within a saltwater recirculation system (water temperature of 20°C, salinity of 18‰, flow rate at 700 L/h (200%/h renewal) and 12 h light/12 h dark photoperiod regime). Levels of total ammonium ($NH_4^+$), nitrite ($NO_2^-$) and nitrate ($NO_3^-$), as well as pH were daily monitored to ensure levels within the recommended ranges for marine species ($NH_4^+ < 0.05$ mg $L^{-1}$; $NO_2^- < 0.5$ mg $L^{-1}$; $NO_3^- < 5$ mg $L^{-1}$; $7.5 < pH \leq 8$). Each diet was randomly assigned to duplicate groups of fish, which were hand fed until visual apparent satiation, three times a day, seven days a week, for 54 days.

Ten fish from the initial fish stock and the 17 fish from each tank at the end of the trial were collected and killed by anaesthetic overdose (MS222, 150 mg $L^{-1}$) after a 48h fasting period. All fish were individually weighed (g) and measured for growth performance evaluation. The 10 fish from the initial stock, and 12 fish from each tank at the end of trial were sampled for whole body composition analysis. Dorsal muscle samples were collected from each fish through a biopsy below the skin (~1 g per fish). For the chemical analysis of both whole body and muscle composition, two independent pools were established per tank, each containing 6 whole fish or 6 muscle samples from those 6 fish. Samples were immediately frozen in dry ice and stored at -80°C for further analysis. Five other fish per tank were collected for instrumental evaluation of skin and muscle colour, and muscle texture. The viscera and liver from these 5 fish were collected and individually weighed to determine the viscerosomatic and hepatosomatic indexes (VSI and HSI, respectively).

## Proximate composition analysis

The experimental diets and whole fish were ground, homogenized and freeze-dried before being further analyzed. Proximate composition analysis followed AOAC (2006) methods: dry matter (in an oven at 105°C for 24 h); ash (incineration in a muffle furnace at 550°C for 6 h; Nabertherm L9/11/B170, Bremen, Germany); crude protein by quantification of nitrogen (N) using a Leco nitrogen analyser (Model FP-528; Leco Corporation, St. Joseph, USA) and conversion (N × 6.25) to equivalent protein; gross energy was determined in an adiabatic bomb calorimetric system (Model Werke C2000, IKA, Staufen, Germany). Total phosphorus content was determined from ash according to AOAC method 965.17 (2006) with the following adaptation: phosphates were quantified after using a 2 mM ammonium heptamolybdate reagent solution with 7 mM ascorbic acid and 0.5 M sulfuric acid at 75 °C in a water bath and later reading the absorbance at 820 nm.

TABLE 2 Ingredients and chemical composition of experimental diets.

| | CTRL | SARDINE | SALMON | ALGARAPE | ALGASOY | ALGABLEND |
|---|---|---|---|---|---|---|
| Ingredients (%) | | | | | | |
| Fishmeal [1] | 15.0 | 15.0 | 15.0 | 15.0 | 15.0 | 15.0 |
| Hydrolyzed fish protein [2] | 3.5 | 3.5 | 3.5 | 3.5 | 3.5 | 3.5 |
| Poultry meal [3] | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 |
| Soy protein concentrate [4] | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 |
| Pea protein concentrate [5] | 5.5 | 5.5 | 5.5 | 5.5 | 5.50 | 5.0 |
| Wheat gluten [6] | 10.0 | 10.00 | 10.0 | 10.0 | 10.0 | 10.0 |
| Corn gluten meal [7] | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 |
| Soybean meal [8] | 6.0 | 6.0 | 6.0 | 6.0 | 6.0 | 6.0 |
| Wheat meal [9] | 14.0 | 14.0 | 14.0 | 14.0 | 14.0 | 12.9 |
| Wheat bran [10] | 5.5 | 5.5 | 5.5 | 5.5 | 5.5 | 5.5 |
| Vitamins and Minerals [11] | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Antioxidant powder [12] | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| Mono-calcium phosphate | 1.1 | 1.1 | 1.1 | 1.1 | 1.1 | 1.1 |
| L-Lysine [13] | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| DL-Methionine [14] | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 |
| Yttrium oxide [15] | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 |
| Sardine oil [16] | 5.0 | 8.5 | - | - | - | 7.9 |
| Salmon oil [17] | - | - | 9.9 | - | - | - |
| Algae oil [18] | - | - | 3.1 | 4.4 | 4.4 | - |
| Soybean oil [19] | - | - | - | - | 8.6 | - |
| Rapeseed oil [20] | 8.0 | 4.5 | - | 8.6 | - | 4.7 |
| Algaessence Feed™ [21] | - | - | - | - | - | 2.0 |
| **Increased cost (%)** | – | 6 | 16 | 17 | 17 | 41 |

*(Continued)*

TABLE 2 Continued

| | CTRL | SARDINE | SALMON | ALGARAPE | ALGASOY | ALGABLEND |
|---|---|---|---|---|---|---|
| Chemical Composition (g or kJ 100g$^{-1}$ DM) | | | | | | |
| Dry matter | 95.3 | 94.8 | 95.0 | 95.1 | 94.6 | 94.8 |
| Protein | 50.6 | 50.3 | 50.6 | 50.9 | 50.6 | 50.1 |
| Crude fat | 17.7 | 17.9 | 18.2 | 17.9 | 18.4 | 18.2 |
| Energy | 23.4 | 23.1 | 23.4 | 23.1 | 23.4 | 23.4 |
| Ash | 7.5 | 7.7 | 7.6 | 7.6 | 7.6 | 7.8 |
| Carbohydrates[22] | 26.8 | 25.6 | 25.7 | 26.1 | 25.7 | 26.2 |
| Total P | 1.1 | 1.1 | 1.1 | 1.1 | 1.1 | 1.1 |

CTRL, Control diet; SARDINE, Sardine oil diet; SALMON, Salmon oil diet; ALGARAPE, Alga and Rapeseed oil diet; ALGASOY, Alga and Soybean oil diet; ALGABLEND, Algaessence diet; DM, dry matter.

[1] Peruvian fishmeal super prime: 71.0% crude protein (CP), 11.0% crude fat (CF), Exalmar, Peru;
[2] Hydrolyzed fish protein, CPSP90: 86% CP, 6% CF, Sopropêche, France;
[3] Poultry meal: 69.1% CP, 13.7% CF, SAVINOR SA., Portugal;
[4] Soy protein concentrate (Soycomil P): 65% CP, 0.7% CF, ADM Animal Nutrition™, The Netherlands;
[5] Pea Protein concentrate: Lysamine GPS, Roquette Frères, France;
[6] VITEN: 82% CP, 2.1% CF, Roquette. France;
[7] Corn gluten meal: 61% CP, 6% CF, COPAM, Portugal;
[8] Soybean meal 44: Cargyll, Spain;
[9] Wheat meal: 10.2% CP, 1.2% CF, Casa Lanchinha, Portugal;
[10] Wheat bran: Casa Lanchinha, Portugal;
[11] Vitamins (IU or mg·kg-1 diet): DL-alpha tocopherol acetate, 255 mg; sodium menadione bisulphate, 10 mg; retinyl acetate, 26000 IU; DL-cholecalciferol, 2500 IU; thiamine, 2 mg; riboflavin, 9 mg; pyridoxine, 5 mg; cyanocobalamin, 0.5 mg; nicotinic acid, 25 mg; folic acid, 4 mg; L-ascorbic acid monophosphate, 80 mg; inositol, 17.5 mg; biotin, 0.2 mg; calcium panthotenate, 60 mg; choline chloride, 1960 mg. Minerals (g or mg·kg$^{-1}$ diet): copper sulphate, 8.25 mg; ferric sulphate, 68 mg; potassium iodide, 0.7 mg; manganese oxide, 35 mg; organic selenium, 0.01 mg; zinc sulphate, 123 mg; calcium carbonate, 1.5 g; excipient wheat middlings; WISIUM, ADM Portugal S.A., Portugal;
[12] VERDILOX. Kemin Europe NV, Belgium;
[13] L-Lisine HCl 99%: Ajinomoto Eurolysine SAS. France;
[14] DL- Methionine 99%: EVONIK Nutrition & Care GmbH. Germany;
[15] Yttrium oxide: Sigma Aldrich, USA;
[16] Sardine oil: Sopropêche, France;
[17] Salmon oil: Sopropêche, France;
[18] Algae oil: Veramaris®, Evonik, NE, USA;
[19] Soybean oil: JCCOIMBRA Distribuição SA., Portugal;
[20] Rapeseed oil: Henry Lamotte Oils GmbH, Germany;
[21] Algaessence Feed™: Blend of Nannocloropsis, Gracilaria and Shizochitrium: 4% CF, ALGAplus Lda., Portugal;
[22] Carbohydrates: Calculated by estimation, 100 – Crude Protein – Crude fat – Ash.

## Total lipids and fatty acid analysis

The experimental diets, whole fish and muscle samples were analysed for total lipids and fatty acids profile.

Total lipids of the experimental diets, whole body and dorsal muscle were extracted and quantified gravimetrically according to Folch et al. (1957) using Folch solution (dichloromethane: methanol 2:1 v/v with 0.01% BHT).

The experimental diets, whole body and muscle lipid extracts were transmethylated by acidic methylation according to Lepage and Roy (1986), with some modifications; adding 3 mL of freshly prepared 5% (v/v) acetyl chloride in anhydrous methanol and heated at 100°C for 1 h. After cooling at room temperature, 5 mL of 6% $K_2CO_3$ solution and 25 mg of BHT dissolved in 2 mL of n-hexane were added. Samples were then vortexed and centrifuged at 700 g for 5 min at room temperature, and the upper organic layer containing fatty acids methyl esters (FAME) was carefully transferred to another tube with 1 g of anhydrous sodium sulphate. This step was repeated twice, before the organic layer was filtered (125 mm Ø) into a new tube, heated at 37°C and dried under a stream of nitrogen gas. Finally, FAME was recovered in 1 mL of n-hexane and analysed in a Shimadzu Nexis GC-2030 gas chromatograph (Kyoto, Japan) equipped with a flame-ionization detector (FID) and a Shimadzu AOC-20i auto-injector. Separation was carried out on an OmegaWax 250 capillary column (30 m× 0.25 mm I.D., film thickness 0.25 µm). Operating conditions were as follows: split mode, with a split ratio of 1:50 and an injection volume of 1 µL. The injector and detector temperatures were kept at 250 and 280°C, respectively. A flow rate of 25 mL min$^{-1}$ of helium as a carrier gas, 40 mL min$^{-1}$ of hydrogen and 400 mL min$^{-1}$ of air were provided. The column thermal gradient was as follows: initial temperature 50°C for 2 min, increased at 50°C min$^{-1}$ to 174°C, hold for 14 min, then increased 2 °C min$^{-1}$ to 210 °C and hold for 50 min. FAME were identified by comparison of retention times with a known standard mixture (Sigma 47,885-U Supelco 37 Component FAME Mix, USA) and quantified using the software GCsolution for GC systems (Shimadzu, Kyoto, Japan). The FAME contents in diets, whole body and muscle were expressed as % of total FAME. The amount of FA expressed in mg 100 g$^{-1}$ of edible part, were calculated using the relative percentage of each peak area (% of total FA) and the lipid conversion factors calculated according to Weihrauch et al. (1977) and Garcia et al. (2019).

TABLE 3 Fatty acid composition of the experimental diets.

| | CTRL | SARDINE | SALMON | ALGARAPE | ALGASOY | ALGABLEND |
|---|---|---|---|---|---|---|
| Fatty acids (% total fatty acids) | | | | | | |
| C14:0 | 2.3 | 3.9 | 2.2 | 1.2 | 1.1 | 4.3 |
| C16:0 (PA) | 11.7 | 14.6 | 14.9 | 15.8 | 16.6 | 14.4 |
| C18:0 | 2.7 | 3.1 | 2.7 | 2.6 | 2.8 | 3.0 |
| **ΣSFA**[1] | 17.7 | 22.8 | 20.6 | 20.7 | 21.5 | 22.8 |
| C16:1 n-7 | 3.4 | 5.5 | 2.3 | 0.9 | 0.8 | 4.9 |
| C18:1 n-9 (OA) | 36.0 | 25.6 | 27.7 | 21.5 | 17.5 | 25.6 |
| C18:1 n-7 | 2.8 | 2.9 | 2.1 | 1.4 | 1.2 | 2.8 |
| C20:1 n-9 | 1.2 | 1.2 | 2.1 | 0.5 | 0.4 | 1.1 |
| **ΣMUFA**[2] | 44.2 | 36.3 | 36.0 | 24.6 | 20.1 | 35.5 |
| C18:2 n-6 (LA) | 17.7 | 12.7 | 15.6 | 30.7 | 35.1 | 14.0 |
| C18:3 n-3 (ALA) | 4.8 | 3.2 | 4.3 | 3.8 | 3.5 | 3.3 |
| C18:4 n-3 | 0.7 | 1.3 | 0.6 | 0.2 | 0.2 | 1.1 |
| C20:4 n-6 | 0.5 | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 |
| C20:5 n-3 (EPA) | 6.1 | 10.5 | 5.8 | 5.3 | 5.0 | 9.4 |
| C22:5 n-3 | 0.7 | 1.0 | 1.2 | 0.6 | 0.6 | 0.9 |
| C22:6 n-3 (DHA) | 3.4 | 4.9 | 10.7 | 11.1 | 11.2 | 6.2 |
| EPA+DHA | 9.5 | 15.4 | 16.5 | 16.4 | 16.2 | 15.6 |
| DHA/EPA | 0.6 | 0.5 | 1.8 | 2.1 | 2.2 | 0.7 |
| Σ n-3[3] | 15.9 | 21.4 | 23.4 | 21.4 | 20.8 | 21.4 |
| Σ n-6[4] | 18.4 | 13.8 | 17.2 | 31.6 | 36.0 | 15.0 |
| Σ n-3/Σ ω6 | 0.86 | 1.55 | 1.36 | 0.68 | 0.58 | 1.43 |
| **ΣPUFA**[5] | 35.5 | 37.2 | 41.1 | 53.2 | 56.9 | 38.6 |
| Fatty acids (g $100g^{-1}$ DM) | | | | | | |
| C20:5 n-3 (EPA) | 1.0 | 1.7 | 1.0 | 0.9 | 0.9 | 1.6 |
| C22:6 n-3 (DHA) | 0.6 | 0.8 | 1.8 | 1.9 | 1.9 | 1.0 |
| EPA+DHA | 1.6 | 2.5 | 2.8 | 2.8 | 2.8 | 2.6 |

CTRL, Control diet; SARDINE, Sardine oil diet; SALMON, Salmon oil diet; ALGARAPE, Alga and Rapeseed oil diet; ALGASOY, Alga and Soybean oil diet; ALGABLEND, Algaessence diet; DM, dry matter; OA, oleic acid; LA, linoleic acid; ALA, α-linolenic acid, EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.
[1] Σ SFA, sum of saturated fatty acids, includes C10:0, C12:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C22:0;
[2] Σ MUFA, sum of monounsaturated fatty acids, includes: C14:1, C16:1, C17:1, C18:1n-7, C18:1n-9, C20:1n-9, C22:1n-9, C22:1n-11, C24:1n-9;
[3] Σ n-3, sum of n-3 PUFA, includes:C18:3n-3, C18:4n-3, C20:3n-3, C20:4n-3, C20:5n-3, C22:5n-3, C22:6n-3;
[4] Σ n-6, sum of n-6 PUFA, includes: C18:2n-6, C18:3n-6, C20:2n-6, C20:3n-6, C20:4n-6;
[5] Σ PUFA, sum of polyunsaturated fatty acids, includes C16:2n-4, C16:3n-4, C16:4n-1, C18:2n-6, C18:3n-6, C18:3n-3, C18:4n-3, C20:2n-6, C20:3n-6, C20:4n-6, C20:3n-3, C20:4n-3, C20:5n-3, C22:5n-3, C22:6n-3.

## Colour and texture

Instrumental measurements of skin and muscle colour were performed at room temperature with a CR-400 Chroma meter (Konica Minolta Inc., Osaka, Japan) with an aperture of 8 mm, at standard illuminant D65 using the CIE 1976 (L*, lightness; *a**, redness; *b**, yellowness). After the calibration of the apparatus with a white plate reference standard, colour parameters were measured by applying the colorimeter onto the raw skin and flesh of the ten fish per dietary treatment (5 fish/tank). Measurements were made above the lateral line in three points of each fillet, and mean values were determined for each fish. After flashing, L*, *a**, and *b** reflected light values were recorded. From a* and b* values, the hue angle ($H° = \tan^{-1} b^*/a^*$) and the Chroma ($C^* = (a^*2 + b^*2)^{1/2}$) were calculated according to Choubert et al. (1997).

Muscle texture was analysed using a TA.XT plus Texture Analyser, with a 5 kg load cell and a 2.0 mm diameter probe (Stable Micro systems Inc., Godalming, UK). Texture profile parameters [hardness (N), adhesiveness (J), springiness (-), cohesiveness (-), chewiness (J), and resilience (-)] were obtained

by double penetration (probe speed of 1 mm $s^{-1}$; probe penetration depth of 4 mm; wait time between penetrations of 5 s) on the thickest part of each raw fillet (Batista et al., 2020).

## Calculations

Growth, feed intake and other somatic parameters were calculated as follows: Daily growth index (DGI) =100 × [(final body weight) $^{1/3}$ - (initial body weight)$^{1/3}$]/days of the experiment; Final condition factor (K) = 100 × (FBW/final body length$^3$); Voluntary feed intake (VFI) = 100 × dry feed intake/average body weight/days of experiment; Feed conversion ratio (FCR) = dry feed intake/weight gain; Protein efficiency ratio (PER) = (FBW − IBW)/ total protein intake (g); HSI = 100 × (liver weight/FBW); VSI = 100 × (weight of viscera/FBW).

Fatty acids utilisation parameters were calculated as follows: FA gain = (FBW × final whole body FA content) – (IBW × initial whole body FA content/ABW/days of experiment; FA retention = 100 × (FBW × final whole body FA content − IBW × initial whole body FA content)/(dry feed intake × FA content in the diet).

Lipid quality indexes were calculated following Ulbricht and Southgate (1991): Atherogenicity index (AI) = [C12:0 + (4 × C14:0) + C16:0] / (Σ PUFAn-6 + Σ PUFAn-3 + Σ MUFA); thrombogenicity index (TI) = (C14:0 + C16:0 + C18:0) / [0.5 × Σ MUFA + 0.5 × Σ PUFAn-6 + 3× Σ PUFAn-3 + (Σ PUFAn-3 / Σ PUFAn-6)]; hypocholesterolemic/hypercholesterolemic FA ratio (h/H) = (C18:1n-9 + C18:2n-6 + C20:4n-6 + C18:3n-3 + C20:5n-3 + C22:5n-3 + C22:6n-3) / (C14:0 + C16:0); peroxidation index (PI) = 0.025 × (% Σ monoenoic FA) + 1 × (% Σ dienoic FA) + 2 × (% Σ trienoic FA) + 4 × (% Σ tetraenoic FA) + 6 × (% Σ pentaenoic FA) + 8 × (% Σ hexaenoic FA).

## Statistical analysis

Statistical analyses were performed using IBM SPSS® Statistics 27.0.1 software (IBM Corporation, Chicago, USA). Data were tested for normality and homogeneity of variances using Kolmogorov-Smirnov and Levene's tests, respectively, followed by a one-way ANOVA. If normality and/or homogeneity of variances assumptions were not met after transformation, a non-parametric test (Kruskal–Wallis H-test) was used instead. When significant differences were observed among dietary treatments, individual means were further compared by a Tukey's multiple comparison test. In all cases, significant differences were considered for a $p < 0.05$.

# Results

## Growth performance, whole body composition and nutrient utilisation

All diets were well accepted by the European sea bass, resulting in similar voluntary feed intake (VFI) (Table 4), and no mortality occurred during the trial. After 54 days of feeding, no significant differences were observed in the fish final body weight (208 – 217g), daily growth index or condition factor. The feed conversion ratio (1.0) and protein efficiency ratio (1.9) remained similar among groups fed the different diets (Table 4). HSI and VSI were not significantly affected by dietary treatments.

The changes in the dietary formulations did not significantly affect sea bass whole body final proximal composition, which remained similar among groups (Table 4). However, whole body total fatty acids profile was strongly affected by the dietary treatments, generally reflecting the dietary FA profile (Table 5; Figure 1). Whole body SFA was significantly higher in fish fed the SARDINE diet than in those fed the CTRL diet, but no other differences were found. The differences found in whole body SFA reflect the differences in palmitic acid (C16:0, PA), which was the most abundant SFA in every diet. MUFA represented the largest FAs fraction in the fish whole body, regardless of the diet (41-48%). Fish fed the CTRL diet had significantly higher MUFA levels than fish fed the diets ALGARAPE and ALGASOY (Table 5), mostly due to high percentage of OA. On the contrary, total PUFA was higher in fish fed ALGASOY or ALGARAPE than in those fed the CTRL or the SARDINE diets (Table 5). The whole body n-3/n-6 ratio was affected by the dietary treatments: the highest ratios (1.1) were observed in fish fed the SARDINE and ALGABLEND diets, and the lowest (0.7) in fish fed the ALGARAPE and ALGASOY diets. The whole-body relative content (% total FA) of either EPA or DHA were highly correlated with their respective dietary levels ($R^2$ >0.98; Figure 1). Whole body retention of individual FA was not significantly affected by the dietary treatments (data not shown; $p > 0.1$), but EPA and DHA gain followed their respective dietary levels (Figure 2). EPA gain increased significantly in fish fed the SARDINE and ALGABLEND diets, whilst DHA showed the highest gain in fish fed the SALMON, ALGARAPE and ALGASOY diets (Figure 2). As a result, all dietary treatments led to a higher EPA +DHA gain when compared to the CTRL diet. The whole body EPA concentration was significantly increased in fish fed the SARDINE and ALGABLEND diets (5.7% of total FA), when compared to ALGARAPE and ALGASOY (4.1% of total FA) (Table 5). On the other hand, the whole body DHA concentration in fish fed SALMON, ALGASOY and ALGARAPE (5.9-6.1% of total FA) doubled that of fish fed the CTRL diet (3.2% of total FA). The highest DHA/EPA ratios were obtained in fish fed SALMON, ALGASOY and ALGARAPE. At the whole body level, fish fed any of the alternative diets had significantly higher EPA+DHA contents than those fed the CTRL diet (1.3 *vs* 1.0g 100$g^{-1}$ wet weight (WW), respectively).

## Muscle lipid content and fatty acid profile

Muscle total lipids were generally low (values ranged between 2.0 and 2.8% WW) and were not significantly affected by the dietary treatments. Muscle FA profile was strongly affected by the fat source and tended to reflect the dietary FA profile (Table 6). Muscle SFA were significantly higher in fish fed the SARDINE, ALGASOY and ALGABLEND diets, when compared to those fed the CTRL diet,

mostly due to the high levels of palmitic acid. When compared to CTRL, the MUFA levels were significantly reduced in the muscle of fish fed ALGASOY, mainly due to the lower concentration of OA. The muscle PUFA was highest in fish fed ALGASOY. The high concentration of LA in the muscle of fish fed the ALGARAPE and ALGASOY resulted in the highest n-6 PUFA levels and lowest n-3/n-6 PUFA ratios (Table 6). The highest muscle EPA concentration was observed in fish fed the SARDINE and ALGABLEND diets, whilst DHA was highest in the muscle of fish fed the SALMON diet. However, the content of EPA+DHA did not vary significantly among diets. All fillets had between 303 and 369 mg EPA+DHA per 100 g wet weight. A linear correlation between the FA profile in the muscle and in the diets was established in Figure 1. A line of equity represented by a solid line corresponds to the alignment of dietary FA levels with tissue fatty acid levels. This line can suggest whether a particularly fatty acid is preferentially retained in a tissue (points above the equity line) or otherwise preferentially metabolized (points below the equity line). Both EPA and DHA tend to be preferentially retained by the muscle, but the opposite was observed in the whole body; EPA and DHA levels in the whole body are lower than those provided by the feed as evidenced by the position against the equity line (Figure 1). EPA was preferentially retained in the muscle of fish fed the ALGASOY, ALGARAPE, SALMON and CTRL diets (Figure 1A; values above the equity line), while DHA was preferentially retained in the muscle of fish fed CTRL, SARDINE, ALGABLEND and SALMON diets (Figure 1B; values above the equity line). Regarding the sum of EPA and DHA (EPA+DHA), the highest muscle retention is observed in fish fed the CTRL, the ALGABLEND, the ALGASOY and the SALMON diets (Figure 1C).

Concerning the lipid quality indexes (Table 6), the atherogenicity index (AI) was significantly higher in fillets from fish fed SARDINE and ALGABLEND when compared to the remaining diets (0.4 *vs.* 0.3, respectively). The hypocolesterolemic and hypercolesterolemic (h/H) FA ratio was higher in the muscle of fish fed the CTRL diet than in those fed with SARDINE, SALMON and ALGABLEND diets (3.4 *vs.* 3.1-3.3 respectively). No significant differences were found in thrombogenicity (TI) and peroxidation (PI) indexes between groups.

## Colour and muscle texture

The skin L*, a* and the Hue (H°) angle were not affected by the dietary treatments (Table 7), but fish fed the SARDINE and ALGABLEND diets were more yellowish (higher b*) and displayed a higher colour saturation (higher C*) than those fed the CTRL diet. Muscle L* and C* were not affected by the dietary treatments, but the muscle of fish fed ALGABLEND had the highest a* and b*. The H° was highest in the muscle of fish fed the ALGARAPE diet and lowest in those fed SALMON and ALGASOY.

Concerning the fillet texture parameters, hardness, cohesiveness and chewiness were similar among groups (Table 7), but significant differences were found in adhesiveness, springiness and resilience. Fish fed the CTRL, SALMON or the ALGABLEND diets displayed a significantly higher muscle adhesiveness than fish fed the ALGARAPE diet. The highest springiness values were found in fish fed the SALMON, ALGARAPE and ALGASOY diets. Muscle of fish fed SALMON and ALGARAPE also had the highest resilience values.

# Discussion

The replacement of fish oil (FO) in aquafeeds implies the identification of alternative and sustainable sources of EPA and DHA, which is nowadays one of the main challenges in aquaculture nutrition, particularly in marine fish species that have limited ability to endogenously synthesize LC-PUFA. Recent research has been focused on finding secure and cost-effective solutions to supply EPA and DHA for commercial fish diets (Tocher, 2015; Tocher et al., 2019; Santigosa et al., 2020; Santigosa et al., 2021; Kousoulaki et al., 2022). In the present study, marine n-3 LC-PUFA rich sources, such as salmon oil, algae oil and a blend of micro- and macroalgae, which are currently available in the market, proved to be viable solutions for directly replacing traditional fish oil (sardine oil - SdO) in aquafeeds. Reasonable combinations were formulated to generate diets that meet the European sea bass dietary requirements for EPA and DHA while providing consumers with differentiated fillets rich in LC-PUFA.

After a short-term growth trial of 54 days, European sea bass juveniles nearly doubled their weight, with no differences in VFI or growth performance between dietary treatments. The data showed that the performance of fish fed diets including AO in combination with either salmon-by products oil or VO was similar to fish fed the CTRL diet and within the range of values previously reported for European sea bass fed diets with FO and FM (Kaushik et al., 2004; Tibaldi et al., 2006). Moreover, neither the dietary content of EPA +DHA (1.6 in CTRL diet *vs* 2.5-2.8 g $100g^{-1}$ DM in the remaining experimental diets), nor the ratio n-3/n-6 (below or above 1), affected fish growth performance, which confirms that the dietary formulations met sea bass requirements for these FA. Despite the differences in the dietary lipid sources, all diets exhibited DHA/EPA ratios between 0.5-2.2, which are within the range of values (0.5-2.0) required for marine fish normal growth and development (NRC, 2011). The total absence of SdO had no significant effect on European sea bass growth, contradicting previous studies reporting growth impairment when VO was used instead of FO (Mourente et al., 2005; Yılmaz et al., 2016). This might be due to the abundance of EPA and DHA in AO that compensates for the deficiencies in VO. Microalgae oil rich in omega-3 has been successfully used without impairing growth or feed efficiency in a number of farmed species (Miller et al., 2007; Qiao et al., 2014; Betancor et al., 2016; Kousoulaki et al., 2020; Santigosa et al., 2020; Santigosa et al., 2021). Likewise, the use of a microalgae's biomass (including *Chlorella* sp., *Tetraselmis* sp. and *Schizochytrium* sp.) to partially replace FM and FO in feeds for gilthead sea bream (*Sparus aurata*) also resulted in similar growth and feed efficiency (Ferreira et al., 2022).

Somatic indexes are good indicators of fat deposition levels, but in the present study, both HSI and VSI remained unaffected by the dietary lipid sources. This is contradictory to what has been

TABLE 4 Growth performance, somatic indexes and whole body composition of European sea bass fed experimental diets.

| | CTRL | SARDINE | SALMON | ALGARAPE | ALGASOY | ALGABLEND | *p*-value |
|---|---|---|---|---|---|---|---|
| **Growth performance** | | | | | | | |
| Initial body weight (g) | 118.1 ± 0.3 | 118.3 ± 1.7 | 118.8 ± 0.1 | 118.9 ± 1.0 | 119.2 ± 0.1 | 118.3 ± 0.5 | 1.000 |
| Final body weight (g) | 211.8 ± 4.4 | 217.1 ± 1.5 | 211.2 ± 6.4 | 215.5 ± 5.7 | 208.0 ± 10.2 | 210.6 ± 4.0 | 0.896 |
| Final Condition Factor | 1.3 ± 0.08 | 1.3 ± 0.004 | 1.3 ± 0.03 | 1.3 ± 0.03 | 1.3 ± 0.1 | 1.3 ± 0.02 | 0.406 |
| Daily Growth Index (DGI) | 2.0 ± 0.04 | 2.0 ± 0.02 | 1.9 ± 0.1 | 2.0 ± 0.07 | 1.9 ± 0.2 | 1.9 ± 0.06 | 0.402 |
| Voluntary Feed Intake (VFI) | 1.1 ± 0.1 | 1.1 ± 0.04 | 1.1 ± 0.03 | 1.1 ± 0.04 | 1.0 ± 0.1 | 1.1 ± 0.1 | 0.620 |
| Feed Conversion Ratio (FCR) | 1.0 ± 0.02 | 1.0 ± 0.03 | 1.0 ± 0.03 | 1.0 ± 0.01 | 1.0 ± 0.02 | 1.0 ± 0.04 | 0.583 |
| Protein Efficiency Ratio (PER) | 1.9 ± 0.04 | 1.9 ± 0.05 | 1.9 ± 0.1 | 2.0 ± 0.01 | 1.9 ± 0.04 | 1.9 ± 0.1 | 0.723 |
| **Somatic indexes (%)** | | | | | | | |
| Hepatosomatic index (HSI) | 1.7 ± 0.001 | 1.7 ± 0.1 | 1.6 ± 0.2 | 1.6 ± 0.0 | 1.9 ± 0.1 | 1.7 ± 0.1 | 0.143 |
| Viscerosomatic index (VSI) | 8.4 ± 0.4 | 8.6 ± 0.5 | 8.5 ± 0.5 | 8.6 ± 0.1 | 8.9 ± 0.5 | 9.0 ± 0.3 | 0.956 |
| **Whole body composition (% or kJ $g^{-1}$ WW)** | | | | | | | |
| Moisture | 61.8 ± 1.25 | 62.4 ± 0.62 | 62.4 ± 1.40 | 61.7 ± 0.34 | 62.9 ± 1.02 | 62.8 ± 0.38 | 0.365 |
| Protein | 16.9 ± 0.46 | 17.0 ± 0.59 | 17.4 ± 0.40 | 16.8 ± 0.25 | 17.1 ± 0.46 | 17.3 ± 0.43 | 0.405 |
| Lipids | 14.7 ± 1.32 | 14.3 ± 0.59 | 13.8 ± 0.66 | 14.5 ± 0.29 | 13.8 ± 0.84 | 13.9 ± 0.45 | 0.379 |
| Energy kJ/g | 10.5 ± 0.35 | 10.4 ± 0.16 | 10.4 ± 0.53 | 10.5 ± 0.13 | 10.4 ± 0.50 | 10.1 ± 0.27 | 0.307 |
| Ash | 3.4 ± 0.11 | 3.2 ± 0.10 | 3.2 ± 0.15 | 3.2 ± 0.34 | 3.2 ± 0.17 | 3.3 ± 0.22 | 0.704 |

CTRL, Control diet; SARDINE, Sardine oil diet; SALMON, Salmon oil diet; ALGARAPE, Alga and Rapeseed oil diet; ALGASOY, Alga and Soybean oil diet; ALGABLEND, Algaessence diet; WW, Wet weight. Values are presented as mean ± standard deviation.
Initial body composition: Moisture, 65.0%; Protein, 17.6% wet weight (WW); Lipids, 11.3% WW; Energy, 9.4kJ $g^{-1}$ WW; Ash, 3.5% WW.

described in several species that showed a tendency for increased HSI and VSI when dietary FO was replaced by VO (Fountoulaki et al., 2009; Bowyer et al., 2012; Willora et al., 2021). However, the present results are consistent with previous findings reported for Atlantic salmon fed diets in which FO was replaced by AO (Miller et al., 2007; Kousoulaki et al., 2022), suggesting that the incorporation of microalgae biomass is a successful strategy to mitigate the negative effects associated with high inclusion of VO.

Although proximate composition remained unaffected by the different lipid sources herein tested, the dietary FA profile clearly influenced the whole-body FA profile. Carnivorous marine species, such as European sea bass, require LC-PUFA in their diets as they have low FA bioconversion rates (Tocher, 2010). The present study shows that the fish capacity to retain n-3 LC-PUFA was not significantly affected by the dietary inclusion level of EPA and DHA. Nevertheless, the percentage of retention varied between 40-51% for EPA and 57-66% for DHA. These values are higher than those previously reported for gilthead seabream (Ferreira et al., 2022) and European seabass (Ferreira et al., 2022; Marques et al., 2022), and they are closer to those obtained for Atlantic salmon fed a FO-based diet, which retentions for EPA and DHA were 30 and 68%, respectively (Ytrestøyl et al., 2015). Moreover, European sea bass fed EPA-rich diets, containing sardine oil (SARDINE and ALGABLEND diets) had a higher EPA gain, while those fed the diets supplemented with AO, particularly rich in DHA (SALMON, ALGARAPE and ALGASOY diets), showed a higher DHA gain (Figure 2A). When compared with the CTRL, all diets promoted EPA+DHA gain, which resulted in a significant increase in fish whole body EPA+DHA content. Still, both fatty acids were metabolized at whole body level, as suggested by their correlation line below the equity line (Figure 1), whilst a selective deposition and retention of these FA was noted in muscle as previously reported in marine fish species, such as Atlantic salmon (Bell et al., 2002; Bell et al., 2003), turbot (Regost et al., 2003), Senegalese sole (Pereira et al., 2019), gilthead seabream (Fountoulaki et al., 2009) and European sea bass (Montero et al., 2005; Marques et al., 2022). In the present study, there is a trend for a higher retention of DHA, as compared to EPA (Figure 2B), which could be due to a higher beta-oxidation of EPA when compared to DHA. EPA retention also seemed to be more influenced by dietary formulation than DHA, but without significant differences among diets (Figure 2B).

From a human nutrition perspective, high n-3 LC-PUFA levels are desirable in muscle due to their health benefits, including a cardio protective effect in adults, improved immune function, cognitive function, mental and metabolic health (Simopoulos, 2000; Harris William et al., 2009). However, the higher the concentration of these FA in the diet, the more tenuous muscle retention seems to be. This was previously described by Rosenlund et al. (2016) who suggested that these FA are increasingly used for metabolism as their dietary concentrations increase. In fact, as displayed by Figure 1., EPA was more metabolized in fish fed ALGABLEND and SARDINE diets (richer in EPA), and DHA in those fed ALGASOY and ALGARAPE diets (richer in DHA),

TABLE 5 Whole body fatty acid composition of the European sea bass fed experimental diets.

| | CTRL | SARDINE | SALMON | ALGARAPE | ALGASOY | ALGABLEND | *p*-value |
|---|---|---|---|---|---|---|---|
| Fatty acids (% total fatty acids) | | | | | | | |
| C14:0 | 2.2 ± 0.05 $^{ab}$ | 2.6 ± 0.04 $^{ab}$ | 2.1 ± 0.03 $^{ab}$ | 1.7 ± 0.03 $^{b}$ | 1.8 ± 0.04 $^{b}$ | 2.8 ± 0.04 $^{a}$ | <0.001 |
| C16:0 | 16.1 ± 0.1 $^{b}$ | 17.6 ± 0.5 $^{a}$ | 17.1 ± 0.1 $^{ab}$ | 17.3 ± 0.4 $^{ab}$ | 17.4 ± 0.5 $^{ab}$ | 16.6 ± 0.2 $^{ab}$ | 0.005 |
| C18:0 | 3.3 ± 0.1 $^{ab}$ | 3.5 ± 0.1 $^{a}$ | 3.2 ± 0.04 $^{b}$ | 3.4 ± 0.1 $^{ab}$ | 3.5 ± 0.2 $^{ab}$ | 3.2 ± 0.1 $^{ab}$ | 0.008 |
| **Σ SFA[1]** | 22.2 ± 0.2 $^{b}$ | 24.4 ± 0.6 a | 23.1 ± 0.1 $^{ab}$ | 23.2 ± 0.5 $^{ab}$ | 23.3 ± 0.7 $^{ab}$ | 23.4 ± 0.3 $^{ab}$ | 0.007 |
| C16:1 n-7 | 4.1 ± 0.04 $^{ab}$ | 4.8 ± 0.05 $^{a}$ | 3.8 ± 0.04 $^{ab}$ | 3.2 ± 0.1 $^{b}$ | 3.1 ± 0.1 $^{b}$ | 4.7 ± 0.1 $^{a}$ | <0.001 |
| C18:1 n-9 (OA) | 38.3 ± 0.6 $^{a}$ | 34.3 ± 0.7 $^{ab}$ | 34.6 ± 0.7 $^{ab}$ | 33.1 ± 0.5 $^{b}$ | 31.3 ± 0.6 $^{b}$ | 34.3 ± 0.2 $^{ab}$ | 0.002 |
| C18:1 n-7 | 2.8 ± 0.02 $^{ab}$ | 2.9 ± 0.02 $^{a}$ | 2.6 ± 0.03 $^{ab}$ | 2.3 ± 0.1 $^{b}$ | 2.2 ± 0.1 $^{b}$ | 2.9 ± 0.04 $^{a}$ | <0.001 |
| C20:1 n-9 | 1.6 ± 0.01 $^{a}$ | 1.5 ± 0.03 $^{ab}$ | 1.8 ± 0.03 $^{a}$ | 1.2 ± 0.1 $^{b}$ | 1.2 ± 0.1 $^{b}$ | 1.5 ± 0.04 $^{ab}$ | <0.001 |
| **Σ MUFA[2]** | 47.7 ± 0.5 $^{a}$ | 44.5 ± 0.7 $^{ab}$ | 43.9 ± 0.7 $^{ab}$ | 40.6 ± 0.7 $^{b}$ | 38.6 ± 0.7 $^{b}$ | 44.4 ± 0.2 $^{ab}$ | 0.001 |
| C18:2 n-6 (LA) | 13.2 ± 0.2 $^{ab}$ | 11.8 ± 0.3 $^{b}$ | 13.3 ± 0.3 $^{ab}$ | 18.1 ± 0.5 $^{a}$ | 19.7 ± 0.5 $^{a}$ | 12.4 ± 0.3 $^{b}$ | <0.001 |
| C18:3 n-3 (ALA) | 3.1 ± 0.05 $^{a}$ | 2.5 ± 0.1 $^{b}$ | 2.9 ± 0.1 $^{a}$ | 2.7 ± 0.1 $^{ab}$ | 2.6 ± 0.1 $^{ab}$ | 2.7 ± 0.1 $^{ab}$ | 0.002 |
| C18:4 n-3 | 0.6 ± 0.01 $^{ab}$ | 0.7 ± 0.01 $^{a}$ | 0.5 ± 0.01 $^{ab}$ | 0.4 ± 0.03 $^{b}$ | 0.4 ± 0.02 $^{b}$ | 0.7 ± 0.01 a | <0.001 |
| C20:4 n-6 | 0.3 ± 0.01 $^{b}$ | 0.4 ± 0.01 $^{ab}$ | 0.4 ± 0.01 $^{ab}$ | 0.4 ± 0.01 $^{ab}$ | 0.4 ± 0.01 $^{ab}$ | 0.4 ± 0.01 $^{a}$ | 0.025 |
| C20:5 n-3 (EPA) | 4.4 ± 0.1 $^{ab}$ | 5.8 ± 0.1 $^{a}$ | 4.3 ± 0.1 $^{ab}$ | 4.1 ± 0.1 $^{b}$ | 4.1 ± 0.1 $^{b}$ | 5.7 ± 0.05 $^{a}$ | <0.001 |
| C22:5 n-3 | 0.6 ± 0.02 $^{ab}$ | 0.7 ± 0.01 $^{ab}$ | 0.8 ± 0.02 $^{a}$ | 0.5 ± 0.01 $^{b}$ | 0.5 ± 0.02 $^{b}$ | 0.7 ± 0.01 $^{ab}$ | <0.001 |
| C22:6 n-3 (DHA) | 3.2 ± 0.1 $^{d}$ | 3.7 ± 0.1 $^{c}$ | 6.0 ± 0.1 $^{a}$ | 5.9 ± 0.2 $^{a}$ | 6.1 ± 0.1 $^{a}$ | 4.4 ± 0.1 $^{b}$ | <0.001 |
| Σ n-3[3] | 12.1 ± 0.3 $^{b}$ | 13.8 ± 0.2 $^{ab}$ | 14.9 ± 0.3 $^{a}$ | 13.9 ± 0.3 $^{ab}$ | 14.1 ± 0.4 $^{ab}$ | 14.5 ± 0.1 $^{a}$ | 0.001 |
| Σ n-6[4] | 14.2 ± 0.3 $^{ab}$ | 12.8 ± 0.3 $^{b}$ | 14.5 ± 0.3 $^{ab}$ | 19.2 ± 0.6 $^{a}$ | 20.9 ± 0.5 $^{a}$ | 13.5 ± 0.3 $^{b}$ | <0.001 |
| Σ n-3/Σ n-6 | 0.9 ± 0.1 $^{c}$ | 1.1 ± 0.2 $^{a}$ | 1.0 ± 0.004 $^{b}$ | 0.7 ± 0.02 $^{d}$ | 0.7 ± 0.01 $^{d}$ | 1.1 ± 0.02 $^{a}$ | <0.001 |
| EPA+DHA | 7.6 ± 0.2 $^{c}$ | 9.5 ± 0.2 $^{b}$ | 10.3 ± 0.2 $^{a}$ | 10.0 ± 0.2 $^{a}$ | 10.2 ± 0.2 $^{a}$ | 10.1 ± 0.1 $^{a}$ | 0.003 |
| DHA/EPA | 0.7 ± 0.01 $^{c}$ | 0.6 ± 0.005 $^{d}$ | 1.4 ± 0.02 $^{b}$ | 1.4 ± 0.1 $^{a}$ | 1.5 ± 0.03 a | 0.8 ± 0.01 $^{c}$ | <0.001 |
| **Σ PUFA[5]** | 27.0 ± 0.5 $^{b}$ | 27.6 ± 0.5 $^{b}$ | 29.9 ± 0.6 $^{ab}$ | 33.5 ± 0.7 $^{a}$ | 35.4 ± 0.9 $^{a}$ | 28.9 ± 0.3 $^{ab}$ | <0.001 |
| Fatty acids (g 100g$^{-1}$ WW) | | | | | | | |
| C20:5 n-3 (EPA) | 0.6 ± 0.05 $^{b}$ | 0.8 ± 0.03 $^{a}$ | 0.6 ± 0.02 $^{bc}$ | 0.5 ± 0.007 $^{bc}$ | 0.5 ± 0.02 $^{c}$ | 0.7 ± 0.02 $^{a}$ | 0.001 |
| C22:6 n-3 (DHA) | 0.4 ± 0.03 $^{c}$ | 0.5 ± 0.02 $^{c}$ | 0.8 ± 0.03 $^{a}$ | 0.8 ± 0.03 $^{a}$ | 0.8 ± 0.04 $^{a}$ | 0.6 ± 0.02 $^{b}$ | 0.001 |
| EPA+DHA | 1.0 ± 0.08 $^{b}$ | 1.3 ± 0.04 $^{a}$ | 1.3 ± 0.05 $^{a}$ | 1.3 ± 0.02 $^{a}$ | 1.3 ± 0.06 $^{a}$ | 1.3 ± 0.04 $^{a}$ | 0.012 |

CTRL, Control diet; SARDINE, Sardine oil diet; SALMON, Salmon oil diet; ALGARAPE, Alga and Rapeseed oil diet; ALGASOY, Alga and Soybean oil diet; ALGABLEND, Algaessence diet; OA, oleic acid; LA, linoleic acid; ALA, α-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. Values are presented as mean ± standard deviation (n=4); Different superscript letters represent significant differences $p < 0.05$.

[1] Σ SFA, sum of saturated fatty acids, includes C10:0, C12:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C22:0;

[2] Σ MUFA, sum of monounsaturated fatty acids, includes: C14:1, C16:1, C17:1, C18:1n-7, C18:1n-9, C20:1n-9, C22:1n-9, C22:1n-11, C24:1n-9;

[3] Σ n-3, sum of n-3 PUFA, includes:C18:3n-3, C18:4n-3, C20:3n-3, C20:4n-3, C20:5n-3, C22:5n-3, C22:6n-3;

[4] Σ n-6, sum of n-6 PUFA, includes: C18:2n-6, C18:3n-6, C20:2n-6, C20:3n-6, C20:4n-6;

[5] Σ PUFA, sum of polyunsaturated fatty acids, includes C16:2n-4, C16:3n-4, C16:4n-1, C18:2n-6, C18:3n-6, C18:3n-3, C18:4n-3, C20:2n-6, C20:3n-6, C20:4n-6, C20:3n-3, C20:4n-3, C20:5n-3, C22:5n-3, C22:6n-3.

although the muscle still reflected fish dietary levels as described in previous studies (Izquierdo et al., 2005; Torrecillas et al., 2017; Santigosa et al., 2021). The balance between mechanisms underlying the selective FA deposition, namely the high specificity of fatty acyl transferases *vs* the relative resistance of these FA to β-oxidation likely explain the similar concentrations of EPA+DHA observed in the muscle (303-369 mg EPA+DHA per 100 g fresh weight). Dietary recommendations for EPA+DHA based on cardiovascular risk prevention for European adults are between 250 and 500 mg day$^{-1}$ according to the European Food Safety Authority (EFSA, 2012). In this study, regardless the dietary EPA+DHA level, all diets were effective in providing fillets with EPA+DHA levels complying with the previously mentioned EFSA recommendations. Santigosa et al. (2020) also reported that the fillet of rainbow trout fed upon a diet with 5% AO had higher EPA+DHA content than that of trout fed a diet including 10% FO. Another study by the same author pointed out that 10% AO is enough to completely replace FO without compromising fillet quality (EPA

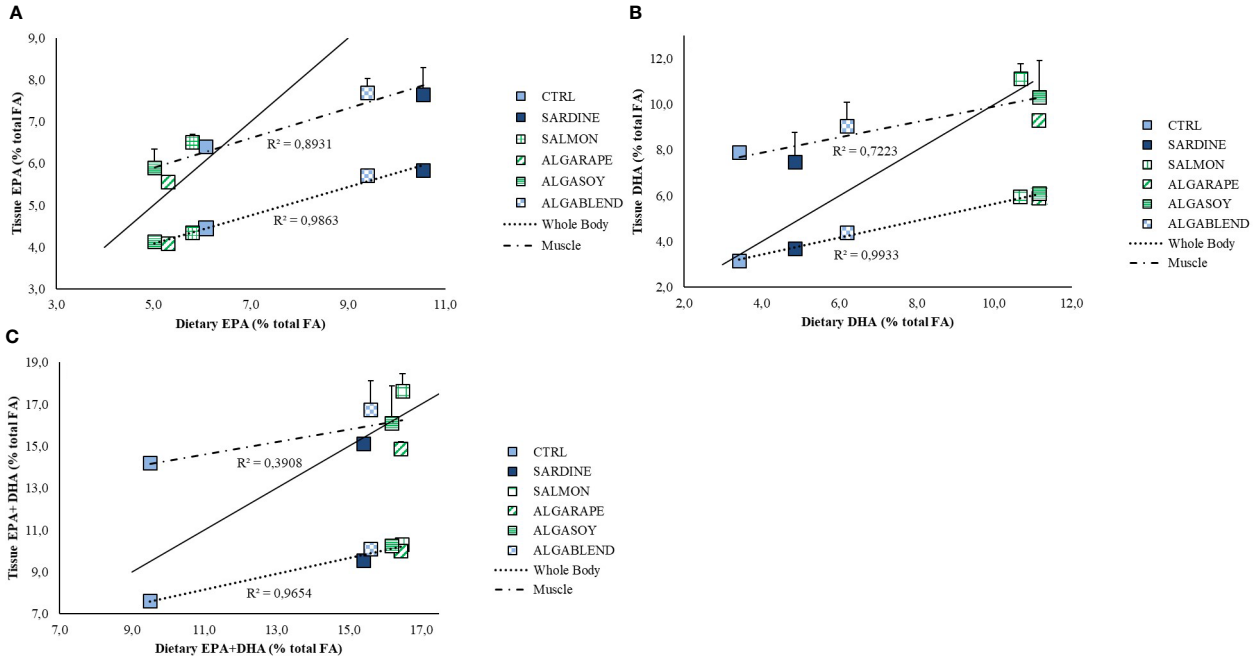


FIGURE 1
Relationship between dietary and whole body or muscle fatty acid content in % total fatty acids for: **(A)** EPA (eicosapentaenoic acid), **(B)** DHA (docosahexaenoic acid) and **(C)** EPA+DHA in European sea bass fed experimental diets for 54 days. A line of equity is represented (solid line). Data is presented as mean ± standard deviation.

+DHA > 250mg $g^{-1}$ wet weight) (Santigosa et al., 2021), results that confirm the ability of Veramaris® oil to totally replace fish oil without compromising flesh n-3 LC-PUFA content. In the present study, when compared to the CTRL, a 10% fortification of sea bass flesh in DHA+EPA was achieved by the SARDINE diet, with a 6% price increase incurred. However, sustainable formulations with a low percentage of AO (<5%), such as ALGARAPE and ALGASOY, allowed for higher fortification (22 and 14%, respectively) at a higher cost (16-17% price increase) but remained viable, highlighting AO dietary inclusion as a feasible solution for sustainable formulations that allow for EPA and DHA fortification.

Other parameters besides total n-3 LC-PUFA have been used to evaluate the nutritional quality of fish for human consumption. The n-3/n-6 and h/H ratio, AI and TI have also been considered as useful indicators of the dietary lipid quality (Ulbricht and Southgate, 1991). In this study it is possible to observe a reduction in muscle n-3/n-6 ratio in the fish fed with either ALGARAPE or ALGASOY diets compared to those fed the remaining diets (1.1 *vs.* 1.4-1.6, respectively), reflecting the respective dietary composition. This was mainly due to the dietary replacement of salmon oil by the blend of RO and SyO, which have a high LA content. VO are rich in LA and contain ALA,

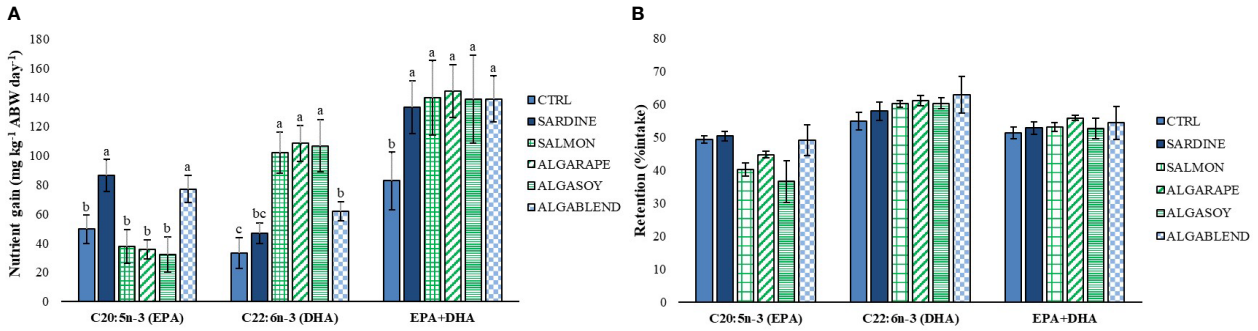


FIGURE 2
**(A)** Whole body gain and **(B)** retention of EPA, DHA and EPA+DHA of European seabass fed experimental diets for 54 days. CTRL, Control diet; SARDINE, Sardine oil diet; SALMON, Salmon oil diet; ALGARAPE, Alga and Rapeseed oil diet; ALGASOY, Alga and Soybean oil diet; ALGABLEND, Algaessence diet; ABW, Average body weight; Values are presented as mean ± standard deviation. Different superscript letters represent significant differences $p < 0.05$.

TABLE 6 Muscle total lipid content, quality indexes and fatty acid composition of the European sea bass fed experimental diets.

| | Initial | CTRL | SARDINE | SALMON | ALGARAPE | ALGASOY | ALGABLEND | *p*-value |
|---|---|---|---|---|---|---|---|---|
| **Total lipids (g 100g$^{-1}$ WW)** | | | | | | | | |
| | 2.1 | 2.5 ± 0.5 | 2.6 ± 0.8 | 2.0 ± 0.2 | 2.8 ± 0.3 | 2.5 ± 0.7 | 2.2 ± 0.4 | 0.288 |
| **Fatty acids (% total fatty acids)** | | | | | | | | |
| C14:0 | 1.8 | 1.7 ± 0.1 $^{b}$ | 2.1 ± 0.1 $^{a}$ | 1.6 ± 0.05 $^{bc}$ | 1.5 ± 0.02 $^{c}$ | 1.4 ± 0.1 $^{c}$ | 2.2 ± 0.1 $^{a}$ | <0.001 |
| C16:0 | 17.0 | 17.0 ± 0.6 $^{b}$ | 17.7 ± 0.1 $^{ab}$ | 18.0 ± 0.3 $^{a}$ | 17.7 ± 0.4 $^{ab}$ | 18.0 ± 0.5 $^{a}$ | 17.5 ± 0.3 $^{ab}$ | 0.016 |
| C18:0 | 4.0 | 4.0 ± 0.1 | 4.2 ± 0.3 | 4.1 ± 0.2 | 4.0 ± 0.1 | 4.3 ± 0.2 | 4.2 ± 0.1 | 0.137 |
| **ΣSFA[1]** | 23.4 | 23.3 ± 0.6 $^{b}$ | 24.6 ± 0.2 $^{a}$ | 24.3 ± 0.4 $^{ab}$ | 23.8 ± 0.5 $^{ab}$ | 24.3 ± 0.6 $^{a}$ | 24.4 ± 0.3 $^{a}$ | 0.006 |
| C16:1 n-7 | 3.2 | 3.4 ± 0.2 $^{bc}$ | 4.0 ± 0.3 $^{a}$ | 3.0 ± 0.1 $^{cd}$ | 2.8 ± 0.2 $^{d}$ | 2.6 ± 0.2 $^{d}$ | 3.7 ± 0.2 $^{ab}$ | <0.001 |
| C18:1 n-9 (OA) | 31.5 | 33.2 ± 1.9 $^{a}$ | 31.3 ± 1.6 $^{ab}$ | 29.9 ± 0.9 $^{ab}$ | 30.0 ± 0.5 $^{ab}$ | 28.1 ± 2.0 $^{b}$ | 30.2 ± 1.0 $^{ab}$ | 0.019 |
| C18:1 n-7 | 2.8 | 2.7 ± 0.1 $^{ab}$ | 2.8 ± 0.04 $^{a}$ | 2.5 ± 0.03 $^{ab}$ | 2.3 ± 0.03 $^{b}$ | 2.3 ± 0.1 $^{b}$ | 2.8 ± 0.03 $^{a}$ | 0.002 |
| C20:1 n-9 | 1.5 | 1.4 ± 0.02 $^{ab}$ | 1.3 ± 0.1 $^{ab}$ | 1.4 ± 0.03 $^{a}$ | 1.1 ± 0.03 $^{b}$ | 1.1 ± 0.1 $^{b}$ | 1.3 ± 0.05 $^{ab}$ | 0.001 |
| **ΣMUFA[2]** | 40.0 | 41.6 ± 2.1 $^{a}$ | 40.2 ± 1.9 $^{ab}$ | 37.8 ± 1.0 $^{bc}$ | 37.0 ± 0.6 $^{bc}$ | 34.8 ± 2.3 $^{c}$ | 38.8 ± 1.3 $^{ab}$ | <0.001 |
| C18:2 n-6 (LA) | 13.3 | 12.5 ± 0.5 $^{ab}$ | 11.2 ± 0.3 $^{b}$ | 11.9 ± 0.2 $^{ab}$ | 16.4 ± 0.4 $^{a}$ | 17.1 ± 0.5 $^{a}$ | 11.4 ± 0.2 $^{b}$ | <0.001 |
| C18:3 n-3 (ALA) | 2.8 | 2.8 ± 0.2 $^{a}$ | 2.4 ± 0.1 $^{b}$ | 2.6 ± 0.03 $^{ab}$ | 2.6 ± 0.1 $^{ab}$ | 2.5 ± 0.1 $^{ab}$ | 2.4 ± 0.1 $^{ab}$ | 0.003 |
| C18:4 n-3 | 0.5 | 0.5 ± 0.04 $^{b}$ | 0.6 ± 0.03 $^{a}$ | 0.44 ± 0.01 $^{bc}$ | 0.39 ± 0.01 $^{cd}$ | 0.37 ± 0.02 $^{d}$ | 0.6 ± 0.03 $^{a}$ | <0.001 |
| C20:4 n-6 | 0.6 | 0.6 ± 0.1 | 0.7 ± 0.1 | 0.8 ± 0.04 | 0.7 ± 0.04 | 0.7 ± 0.1 | 0.8 ± 0.1 | 0.092 |
| C20:5 n-3 (EPA) | 6.8 | 6.4 ± 0.1 $^{bc}$ | 7.6 ± 0.7 $^{a}$ | 6.5 ± 0.2 $^{b}$ | 5.6 ± 0.1 $^{c}$ | 5.9 ± 0.5 $^{bc}$ | 7.7 ± 0.3 $^{a}$ | 0.001 |
| C22:5 n-3 | 0.9 | 0.85 ± 0.08 $^{ab}$ | 0.91 ± 0.06 $^{a}$ | 0.97 ± 0.05 $^{a}$ | 0.72 ± 0.01 $^{c}$ | 0.76 ± 0.07 $^{bc}$ | 0.93 ± 0.03 $^{a}$ | <0.001 |
| C22:6 n-3 (DHA) | 7.9 | 7.9 ± 2.1 $^{b}$ | 7.5 ± 1.3 $^{b}$ | 11.1 ± 0.6 $^{a}$ | 9.3 ± 0.2 $^{ab}$ | 10.3 ± 1.6 $^{ab}$ | 9.1 ± 1.0 $^{ab}$ | 0.007 |
| Σ n-3[3] | 19.2 | 18.6 ± 2.1 | 19.3 ± 1.9 | 22.0 ± 0.9 | 18.8 ± 0.4 | 19.9 ± 2.1 | 21.0 ± 1.3 | 0.053 |
| Σ n-6[4] | 14.5 | 13.8 ± 0.4 $^{ab}$ | 12.5 ± 0.3 $^{b}$ | 13.5 ± 0.2 $^{ab}$ | 17.8 ± 0.4 $^{a}$ | 18.6 ± 0.5 $^{a}$ | 12.8 ± 0.2 $^{b}$ | <0.001 |
| Σ n-3/Σ n-6 | 1.3 | 1.4 ± 0.2 $^{ab}$ | 1.5 ± 0.1 $^{ab}$ | 1.6 ± 0.1 $^{a}$ | 1.1 ± 0.02 $^{b}$ | 1.1 ± 0.1 $^{b}$ | 1.6 ± 0.1 $^{a}$ | 0.003 |
| EPA+DHA | 14.7 | 13.2 ± 0.4$^{b}$ | 15.1 ± 1.9$^{ab}$ | 17.6 ± 0.8$^{a}$ | 14.9 ± 0.3$^{ab}$ | 16.9 ± 1.7$^{a}$ | 16.8 ± 1.4$^{a}$ | 0.030 |
| DHA/EPA | 1.2 | 1.2 ± 0.3 $^{ab}$ | 1.0 ± 0.1 $^{b}$ | 1.7 ± 0.1 $^{a}$ | 1.7 ± 0.01 $^{ab}$ | 1.8 ± 0.1 $^{a}$ | 1.17 ± 0.08 $^{ab}$ | 0.005 |
| **ΣPUFA[5]** | 34.3 | 33.0 ± 1.9 $^{c}$ | 32.5 ± 2.0 $^{c}$ | 36.1 ± 1.1 $^{abc}$ | 37.0 ± 0.9 $^{ab}$ | 38.9 ± 2.4 $^{a}$ | 34.4 ± 1.3 $^{bc}$ | <0.001 |
| **Fillet quality indexes** | | | | | | | | |
| AI | 0.03 | 0.3 ± 0.01$^{b}$ | 0.4 ± 0.01$^{a}$ | 0.3 ± 0.01$^{b}$ | 0.3 ± 0.01$^{b}$ | 0.3 ± 0.01$^{b}$ | 0.4 ± 0.01$^{a}$ | <0.001 |
| TI | 0.27 | 0.3 ± 0.01 | 0.3 ± 0.02 | 0.3 ± 0.01 | 0.3 ± 0.01 | 0.3 ± 0.02 | 0.3 ± 0.01 | 0.106 |
| h/H | 3.4 | 3.4 ± 0.09 $^{a}$ | 3.1 ± 0.04 $^{c}$ | 3.3 ± 0.08$^{bc}$ | 3.4 ± 0.10 $^{ab}$ | 3.4 ± 0.09$^{ab}$ | 3.2 ± 0.03 $^{c}$ | <0.001 |
| PI | 136.9 | 133.3 ± 17.4 | 137.0 ± 14.5 | 160.9 ± 7.2 | 141.6 ± 3.3 | 152.1 ± 16.8 | 150.3 ± 10.3 | 0.055 |
| **Fatty acids (mg 100g$^{-1}$ WW)** | | | | | | | | |
| C20:5 n-3 (EPA) | 122.5 | 137.9 ± 26.0 | 170.0 ± 40.9 | 111.8 ± 6.5 | 138.2 ± 12.4 | 126.2 ± 27.2 | 143.2 ± 24.2 | 0.101 |
| C22:6 n-3 (DHA) | 143.0 | 165.6 ± 16.2 $^{b}$ | 163.1 ± 26.6 $^{b}$ | 190.7 ± 6.1 $^{ab}$ | 231.0 ± 19.9 $^{a}$ | 218.2 ± 31.7 $^{a}$ | 166.7 ± 18.9 $^{b}$ | <0.001 |
| EPA+DHA | 265.4 | 303.5 ± 30.0 | 333.1 ± 67.5 | 302.5 ± 12.4 | 369.2 ± 32.3 | 344.3 ± 58.7 | 310.0 ± 42.9 | 0.269 |

CTRL, Control diet; SARDINE, Sardine oil diet; SALMON, Salmon oil diet; ALGARAPE, Alga and Rapeseed oil diet; ALGASOY, Alga and Soybean oil diet; ALGABLEND, Algaessence diet; OA, oleic acid; LA, linoleic acid; ALA, α-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; AI, Atherogenicity index; TI, Thrombogenicity index; h/H, Hypocholesterolemic/hypercholesterolemic FA ratio; PI, Peroxidation index; Values are presented as mean ± standard deviation (n=4); Different superscript letters represent significant differences $p < 0.05$.

[1] Σ SFA, sum of saturated fatty acids, includes C10:0, C12:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C22:0;

[2] Σ MUFA, sum of monounsaturated fatty acids, includes: C14:1, C16:1, C17:1, C18:1n-7, C18:1n-9, C20:1n-9, C22:1n-9, C22:1n-11, C24:1n-9;

[3] Σ n-3, sum of n-3 PUFA, includes:C18:3n-3, C18:4n-3, C20:3n-3, C20:4n-3, C20:5n-3, C22:5n-3, C22:6n-3;

[4] Σ n-6, sum of n-6 PUFA, includes: C18:2n-6, C18:3n-6, C20:2n-6, C20:3n-6, C20:4n-6;

[5] Σ PUFA, sum of polyunsaturated fatty acids, includes C16:2n-4, C16:3n-4, C16:4n-1, C18:2n-6, C18:3n-6, C18:3n-3, C18:4n-3, C20:2n-6, C20:3n-6, C20:4n-6, C20:3n-3, C20:4n-3, C20:5n-3, C22:5n-3, C22:6n-3.

TABLE 7 Flesh texture (TPA) and skin colour in European sea bass fed the experimental diets.

| | CTRL | SARDINE | SALMON | ALGARAPE | ALGASOY | ALGABLEND | *p*-value |
|---|---|---|---|---|---|---|---|
| **Skin instrumental colour** | | | | | | | |
| *L** | 46.4 ± 11.4 | 56.6 ± 7.1 | 57.8 ± 7.2 | 51.2 ± 10.6 | 50.1 ± 9.5 | 51.0 ± 5.7 | 0.055 |
| *a** | -0.3 ± 0.5 | -0.4 ± 0.5 | -0.4 ± 0.3 | -0.1 ± 0.5 | -0.2 ± 0.4 | -0.4 ± 0.5 | 0.734 |
| *b** | 5.8 ± 2.4 $^{b}$ | 9.1 ± 1.6 $^{a}$ | 7.7 ± 1.3 $^{ab}$ | 7.0 ± 2.5 $^{ab}$ | 6.4 ± 2.0 $^{ab}$ | 8.9 ± 2.3 $^{a}$ | 0.004 |
| *C** | 5.9 ± 2.4 $^{b}$ | 9.1 ± 1.6 $^{a}$ | 7.7 ± 1.3 $^{ab}$ | 7.0 ± 2.5 $^{ab}$ | 6.4 ± 2.0 $^{ab}$ | 8.9 ± 2.3 $^{a}$ | 0.004 |
| *H°* | 92.7 ± 5.3 | 92.0 ± 3.5 | 93.4 ± 1.2 | 90.6 ± 4.0 | 92.1 ± 3.9 | 92.4 ± 3.3 | 0.61 |
| **Muscle instrumental colour** | | | | | | | |
| *L** | 38.4 ± 1.3 | 38.3 ± 0.7 | 39.2 ± 1.1 | 37.8 ± 0.9 | 38.8 ± 1.0 | 38.2 ± 1.3 | 0.118 |
| *a** | -0.6 ± 0.04 $^{ab}$ | -0.8 ± 0.2 $^{ab}$ | -0.9 ± 0.4 $^{b}$ | -0.7 ± 0.1 $^{ab}$ | -0.9 ± 0.2 $^{b}$ | -0.5 ± 0.1 $^{a}$ | 0.004 |
| *b** | -1.3 ± 0.7 $^{ab}$ | -0.8 ± 0.6 $^{ab}$ | -0.8 ± 0.6 $^{ab}$ | -1.4 ± 0.3 $^{b}$ | -0.8 ± 0.7 $^{ab}$ | -0.6 ± 0.5 $^{a}$ | 0.003 |
| *C** | 1.5 ± 0.6 | 1.2 ± 0.3 | 1.3 ± 0.5 | 1.5 ± 0.2 | 1.3 ± 0.4 | 1.0 ± 0.4 | 0.09 |
| *H°* | 240.6 ± 8.5 $^{ab}$ | 227.5 ± 11.8 $^{abc}$ | 221.3 ± 17.0 $^{bc}$ | 246.6 ± 10.8 $^{a}$ | 214.8 ± 24.4 $^{c}$ | 233.1 ± 17.9 $^{abc}$ | 0.001 |
| **Muscle instrumental texture** | | | | | | | |
| Hardness (N) | 0.7 ± 0.1 | 0.7 ± 0.1 | 0.7 ± 0.1 | 0.7 ± 0.1 | 0.7 ± 0.1 | 0.6 ± 0.4 | 0.077 |
| Adhesiveness (J) | -0.03 ± 0.01 $^{a}$ | -0.05 ± 0.03 $^{ab}$ | -0.05 ± 0.07 $^{a}$ | -0.15 ± 0.12 $^{b}$ | -0.12 ± 0.13 $^{ab}$ | -0.05 ± 0.06 $^{a}$ | <0.001 |
| Springiness | 0.99 ± 0.07 $^{b}$ | 0.98 ± 0.03 $^{b}$ | 1.09 ± 0.22 $^{a}$ | 1.08 ± 0.12 $^{a}$ | 1.08 ± 0.17 $^{a}$ | 1.01 ± 0.05 $^{ab}$ | <0.001 |
| Cohesiveness | 0.45 ± 0.05 | 0.45 ± 0.07 | 0.46 ± 0.06 | 0.43 ± 0.03 | 0.44 ± 0.05 | 0.44 ± 0.05 | 0.215 |
| Chewiness (J) | 0.35 ± 0.08 | 0.32 ± 0.12 | 0.33 ± 0.08 | 0.29 ± 0.05 | 0.29 ± 0.16 | 0.26 ± 0.17 | 0.058 |
| Resilience | 0.31 ± 0.18 $^{ab}$ | 0.24 ± 0.05 $^{b}$ | 0.34 ± 0.11 $^{a}$ | 0.38 ± 0.19 $^{a}$ | 0.34 ± 0.19 $^{ab}$ | 0.25 ± 0.07 $^{b}$ | <0.001 |

CTRL, Control diet; SARDINE, Sardine oil diet; SALMON, Salmon oil diet; ALGARAPE, Alga and Rapeseed oil diet; ALGASOY, Alga and Soybean oil diet; ALGABLEND, Algaessence diet; Values are presented as mean ± standard deviation (n=10); Different superscript letters represent significant differences $p < 0.05$.
* refers to a vector.

but totally lack EPA and DHA, unbalancing its n-3/n-6 ratio towards values lower than 1. Therefore, high FO replacement levels by VO may decrease the benefits of eating fish as suggested by Simopoulos (2012), not only by decreasing EPA and DHA levels in muscle, but also because excessive intake of n-6 PUFA and consequently low n-3/n-6 ratio can have adverse health effects promoting the development/appearance of cardiovascular diseases, cancer, osteoporosis and inflammatory and autoimmune diseases (Okuyama et al., 1996; Simopoulos, 2016; Yue et al., 2021). Such FA imbalances in muscle derived from low dietary n-3/n-6 ratio might also inhibit the biosynthesis of the physiologically active n-3 LC-PUFA and hence decrease the EPA and DHA deposition levels (Power and Newsholme, 1997; Bandarra et al., 2011). Although in the present study the total replacement of sardine oil by a blend of VO and AO did not impair EPA and DHA muscle levels, these fish still had the lowest n-3/n-6 ratio. Likewise, muscle h/H ratio was affected by the experimental diets being lower in the fish fed the SARDINE, SALMON and ALGABLEND diets (h/H = 3.1 to 3.3) comparatively to the remaining. This was mainly associated with the higher content of SFA in the muscle of these fish, namely the myristic (C14:0) and palmitic acids (C16:0), resulting from a large abundance of these FA in the ingredients included in the testing diets, such as sardine oil (6.7% C14:0 and 16.7% of C16:0) and the algae's blend (31.3% C14:0 and 15.7% of C16:0). To the best of our knowledge, there are no reference values for h/H ratio, but as suggested by Santos-Silva et al. (2002) and according to the current knowledge on the effects of specific FA on cholesterol metabolism, higher values of h/H should be considered as positive. Comparing the present results on h/H ratio with those reported in a previous study by Testi et al. (2006) in seawater-reared European sea bass selected from stocks of ready-for-sale fish (h/H = 2.1), all fish fed the experimental diets herein tested had higher h/H ratios.

Atherogenicity (AI) and thrombogenicity index (TI) are indicators of the potential impact of food on cardiovascular health. AI is defined by the ratio between the sum of the main SFA and MUFA, the first being considered pro-atherogenic (Σ SFA), and the latter anti-atherogenic (Σ PUFA n-6 + Σ PUFA n-3 + Σ MUFA), whilst TI indicates a propensity for clot formation in blood vessels and it is defined as the relationship between the pro-thrombogenetic and the anti-thrombogenetic FA (Σ SFA and Σ PUFA n-6 + Σ PUFA n-3 + Σ MUFA, respectively) (Ulbricht and Southgate, 1991). Although lower AI and TI values indicate higher nutritional quality and greater potential for reducing the risk of coronary heart disease, no recommended values for IA and IT have been established yet (Chen and Liu, 2020). Furthermore, several factors such as environmental conditions, fish weight and fish age may affect these indexes, but they seem to be predominantly related

to diet, which is known to impact the FA profile (Grigorakis, 2007). The TI values herein obtained (0.2-0.3) were not affected by dietary treatments and were in line with those reported for same-sized cultured European sea bass (Alasalvar et al., 2002; Testi et al., 2006). On the other hand, the highest AI values were found in the fillets of fish fed SARDINE and ALGABLEND diets, which had sardine oil as main lipid source. Grigorakis (2007) reported even higher AI values (0.6) in market-sized European sea bass fed a FO-diet. Moreover, Álvarez et al. (2020) found that replacing FO with VO (soybean and rapeseed oils) in gilthead seabream (*Sparus aurata*) led to decreased AI values, which is consistent with the present data. In conclusion, replacing dietary sardine oil by alternative lipid sources can decrease AI and potentially enhance flesh fat quality. Nevertheless, it is important to keep in mind that fish still have lower AI and TI values compared to other food sources, making them an excellent choice for maintaining heart health (Olmedilla-Alonso et al., 2002).

Lipid peroxidation index (PI), represent the relationship between the FA composition of a tissue and its susceptibility to oxidation, being PUFA more sensitive to oxidation than MUFA (Hulbert et al., 2007). Despite the higher PUFA content in the muscle of fish fed the diets including AO (SALMON, ALGARAPE and ALGASOY), PI was not significantly affected. However, the flesh of fish fed the SALMON diet displayed the highest PI, which seems to indicate a higher susceptibility for FA oxidation. Further analysis throughout post-mortem could clarify the extent of the impact of dietary lipid sources on lipid oxidation.

The organoleptic quality of a final product is just as important as its nutritional value because it can influence a consumer's purchase decision (Ramalho Ribeiro et al., 2015; Álvarez et al., 2020; Batista et al., 2020). In this study, fish fed the ALGABLEND diet showed increased colour saturation (more precisely the b*) and a more yellowish skin. Likewise, the fillet of fish fed the ALGABLEND diet had a tendency towards a more intense red (higher a* value) and yellow (higher b* value) colour, without differing significantly from the CTRL. Batista et al. (2020) also reported higher a* values in fillets of European sea bass fed with a diet containing 8% of the algae *Gracilaria* sp. These findings suggest that algae, being good sources of pigments, could improve the appearance of fish. Texture is also one of the most important quality attributes of fish and meat. It contributes to consumer acceptance and therefore marketability of the final product (Bland et al., 2018). Hardness is probably the most easily textural parameter perceived by consumers that generally appreciate a high muscle hardness (Cheng et al., 2014). In this study, it was found that SdO could be totally replaced by different reasonable combinations of alternative lipid sources without altering flesh hardness. This could be partly related to the absence of significant differences in muscle lipid and moisture contents, which are important chemical variables for flesh texture (Johnston et al., 2006; Xu et al., 2021). Moreover, springiness was significantly increased in diets containing AO, and resilience was lowest in fish fed with SARDINE and ALGABLEND diets (SdO inclusion > 7.9%). However, longer feeding trials and further validation by a sensorial panel are needed to fully determine the impact of alternative lipid sources on sea bass nutritional and sensory properties.

## Conclusion

In this study, various marine n-3 LC-PUFA rich sources (salmon oil, algae oil and a blend of micro and macroalgae, Algaessence Feed$^{TM}$), currently available in the market, were demonstrated to be viable solutions for the direct replacement of a traditional fish oil (sardine oil - SdO) in diets for European sea bass, contributing to the environmental and economical sustainability of aquaculture. After a 54 days' trial, all diets promoted fish growth equally and provided consumers differentiated fillets rich in EPA+DHA, with over 250 mg of EPA+DHA per 100 g fillet complying with EFSA recommended daily intakes to reduce the risk of cardiovascular diseases (>250 mg day$^{-1}$), and without major impacts on texture. Still, the flesh of fish fed ALGARAPE and ALGASOY had the highest DHA concentrations due to the dietary inclusion of a DHA rich algae oil. SALMON and ALGABLEND fed fish displayed the highest PUFA n-3/n-6 ratios. Overall, this study showed that combining pricy sources of n-3 LC-PUFA (Veramaris® or Algaessence Feed$^{TM}$) with less expensive oils (salmon by-products oil or vegetable oils) was a successful strategy to fortify European sea bass flesh with EPA and DHA while mitigating the negative effects associated with high dietary inclusions of vegetable oils. However, based on FA retention, there was no apparent advantage in increasing dietary n-3 LC-PUFA in European sea bass diets, as there appears to be a threshold above which β-oxidation seems to be promoted. Future studies are required to determine the most adequate dietary levels of EPA and DHA to promote high retentions in large-sized European sea bass while ensuring desirable nutritional flesh quality and the cost-effectiveness of aquafeeds.

## Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

## Ethics statement

Fish trials were conducted in compliance with the guidelines of the European Union (Directive 2010/63/EU) and approved by the Ethical Committee of Riasearch Lda., Murtosa, Portugal, overseen by the National Competence Authority. The study was conducted in accordance with the local legislation and institutional requirements.

## Author contributions

AM: Methodology, validation, formal analysis, investigation, validation and writing review and editing. PC: validation and

writing review. CC and FP: investigation and formal analysis. AB: investigation and writing review. MS: formal Analysis, funding acquisition and writing review. HA and JD: conceptualization and funding Acquisition. LV: conceptualization, methodology, resources, writing review & editing, supervision, project administration and funding acquisition. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

Author HA was employed by the company Produção e Comercialização de Algas e seus Derivados Lda. and author JD was employed by the companies SPAROS Lda. and RIASEARCH Lda.

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.

The reviewer DF declared a past co-authorship with the author LV to the handling editor.

## Publisher's note



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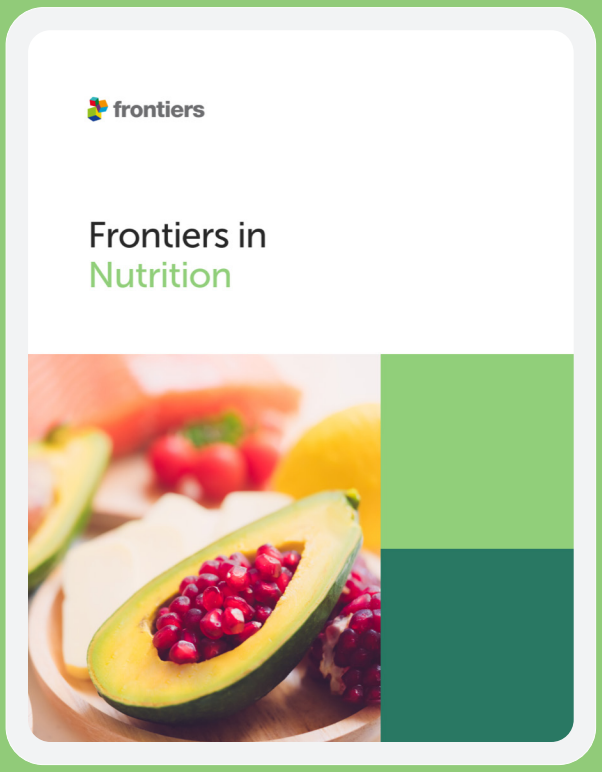


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