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Use of flavorings to improve the palatability of aquafeeds used in aquaculture: a multidisciplinary approach to better understand teleost physiological responses

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Chapter 1

Introduction

1.1 Overview of the aquaculture sector and the use of alternative protein sources: benefits and disadvantages for fish diets

Aquaculture is defined as the rearing of aquatic organisms, such as fish, mollusks, crustaceans, and aquatic plants, utilizing diverse methodologies, ranging from traditional pond culture to intensive systems which employ advanced technologies (Lucas & Southgate, 2012). Nowadays, aquaculture is an ever-expanding sector, which requires to meet the increasing global demand of seafood. In 2022, world aquaculture production reached an all-time record of 130.9 million tons, being the first time in history that global aquaculture production of animal species surpassed capture fisheries production (FAO, 2024). In the same year, total world aquaculture production grew by 87.9 million tons compared to 43 million tons in 2000, with an average annual growth rate of 5.2%. Human fish consumption has more than doubled in the last 50 years, reaching 20.2 kg per capita in 2020, accounting for about 16% of animal protein consumption by human (FAO, 2022; Hussain et al., 2024). In this context, the growing demand of an ever-expanding population, which expects to reach 9.7 billion people by 2050, requires a sustainable development of the sector, especially in farming practices (Singh et al., 2023). Indeed, although aquaculture has been promoted as a solution to overfishing, at the same time it is one of the causes of the decline in wild fish populations, as it partially depends on capture fisheries to provide fishmeal for aquafeeds (Ahmed & Thompson, 2019).

Fishmeal (FM) is a commonly used ingredient to feed aquatic animals, which consists in a protein-rich meal made by milling and drying whole fish, fish trimmings, or other fish by-products (Hussain et al., 2024). Two basic types of FM exist, which are either manufactured from by-products of seafood-processing industries, or derived from small, wild-caught bony fish, specifically harvested to produce FM, that are otherwise not suitable for human consumption (Cho & Kim, 2011). The main species used for FM production are small pelagic fish, such as Peruvian anchovy (which accounts for the largest percentage), menhaden, blue whiting, capelin, shad, sardine, mackerel and herring (FAO, 2024).

From a nutritional standpoint, FM is a source of high-quality protein with a balanced amino acid profile, omega-3 polyunsaturated fatty acids (PUFA), minerals, vitamins (such as biotin, choline, vitamin A, D, E, and B12), and trace elements like iodine and selenium (Cho & Kim, 2011; Hussain et al., 2024). High-quality FM typically contains between 60% and 72% crude protein, and lipids ranging from 6–10% or 4–20% by weight, with essential amino acids content. It is highly digestible and palatable and provides large quantities of energy per unit weight.

Even though FM is an ideal protein source for formulating fish diets, its use has become unsustainable from both economic and environmental perspectives. In fact, due to overfishing, which has led to a decline in marine

species, FM is nowadays a limiting factor, which cannot be employed as a sole source of protein within feed (Hussain et al., 2024; S. Zhou et al., 2015). Moreover, the rapid growth of aquaculture has additionally increased the requirement for protein sources, driving up the FM demand. As a consequence, FM prices have exponentially increased in recent years, and aquafeeds currently account for more than 50% of aquaculture production costs (Beal et al., 2018; Ye et al., 2020).

To date, FM is selectively used at specific production phases, such as hatchery, broodstock and finishing diets, while its incorporation in grow-out diets is declining. For example, its inclusion level in the grow-out diets for Atlantic salmon is now often less than 10 percent, with a continuous reduction across all species (FAO, 2024).

In a circular economic context, aquaculture requires a sustainable development of the sector, thereby employing tailored feeding strategies which encompass the use of more sustainable and cost-effective ingredients for aquafeed formulations.

Plant-derived ingredients

Plant-based protein sources represent a significant progress in the development of more sustainable aquafeed formulations, which are classified as Aquafeed v2.0 (Eroldoğan et al., 2023). Vegetable sources are widely employed in aquaculture mainly due to their higher availability and low prices when compared to FM (Gasco et al., 2018; Mugwanya et al., 2023). In recent decades, soybean has become the most commonly used among vegetable ingredients in substituting FM, followed by cereal glutens, such as corn and wheat, pulses, and vegetable protein concentrates (Gatlin et al., 2007; Hameed et al., 2022). However, the use of plant-derived meals presents relevant limitations related to the feed-food-fuel competition, which affects their availability and cost, competing with human needs (Singh et al., 2023). At the same time, adverse health effects occur in carnivorous species when they are incorporated at high percentages within diets (Gaudioso et al., 2021; J. S. Zhou et al., 2018). In fact, the presence of anti-nutritional factors (ANFs) and undigestible carbohydrates in vegetable meals often negatively affects their digestibility and nutrient assimilation, leading to inflammation of the fish gastrointestinal tract (Gatlin et al., 2007). For instance, non-starch polysaccharides, and other undigestible carbohydrates (like fibers) often affect nutrient utilization thereby reducing the overall feed efficiency (Bandara, 2018; Francis et al., 2001).

Among ANFs, antigenic compounds, protease inhibitors, phytates, tannins, lectins, phytic acid, and saponins are the major cause of reduced feed intake and growth (Francis et al., 2001; Gatlin et al., 2007). Moreover, it is worth noting that the unbalanced amino acid profile of vegetable meals, lacking the essential amino acids necessary for fish health (e.g., methionine and lysine), often makes necessary either dietary amino acids supplementation or a combination with other ingredients (Aragão et al., 2022). Only a few studies suggest that complete FM replacement by plant-derived meals did not affect the fish's zootechnical performances (Daniel, 2018; Yun et al., 2014; Zhang et al., 2012). For instance, Snyder et al., (2012) demonstrated that a plant-based diet, completely deprived in FM, reduced muscle growth and increased muscle protein

degradation in rainbow trout (*Oncorhynchus mykiss*), over a 9-week trial. Gómez-Requeni et al., (2004) demonstrated that, despite large fish meal replacements (50–75%) are feasible in marine finfish diets, a complete replacement of FM (100%) depresses weight gain in gilthead sea bream (*Sparus aurata*), because of a marked reduction of feed intake.

The digestion of plant-based meals often affects the health and integrity of both intestine and liver of the fish (Krogdahl et al., 2010). Especially in carnivorous species, high dietary inclusion of plant-derived meal induces enteritis, compromising the integrity of the intestinal epithelium due to inflammation events (Gu et al., 2016; Merrifield et al., 2011). This negatively affects the proper nutrient absorption process, leading to secretory phenomena as a pathological state of the intestine, and impair digestive functions eventually reducing feed conversion ratio (FCR) and growth (Krogdahl et al., 2010). Santigosa et al. (2011) demonstrated that intestinal nutrient absorption was modified in both rainbow trout and gilthead sea bream in response to the use of high levels of plant protein sources. Other studies on carnivorous species such as gilthead seabream (Baeza-Ariño et al., 2016), senegalese sole (*Solea senegalensis*) (Batista et al., 2016), rainbow trout , and Atlantic salmon (*Salmo salar*) (Buttle et al., 2001; Rumsey et al., 1994) evidenced that the inclusion of high levels of plant ingredients affected the fish health conditions, including an impairment in the immune and inflammatory responses, as well as of the gut and liver morphology, evidencing pathological changes. Complementing this, plant ANFs can also alter tissue morphology and cause steatosis, contributing to metabolic dysfunction that have further negative consequences on fish zootechnical performance (Martínez-Llorens et al., 2012).

Several procedures have been implemented during aquafeeds production to improve the digestibility of vegetable meals and nutrient absorption. Several technical processing steps during ingredient production, such as protein alcohol extraction, fermentation, or heat can inactivate or reduce the content of the ANFs (Glencross, Baily, et al., 2020). For instance, heat labile factors, including trypsin inhibitor and lectins, can be eliminated or reduced by heat treatment during processing of soybean meal (Drew et al., 2007). Moreover, protease application allows the hydrolysis of proteinaceous antinutrients which are known to be harmful (S. Chen et al., 2024). Other solutions include the addition of essential amino acids (EAAs) (Goda et al., 2007), the aggregation of different plant sources (Liti et al., 2006), the application of exogenous enzymes (e.g., supplementation of phytase to increase P absorption from plant meals) (T. T. Jiang et al., 2014), the alternation of plant-based and FM-based diets (Nandeesha et al., 2002), and the inclusion of feed supplements (R. B. Johnson et al., 2015). For example, the incorporation of additives such as butyrate can mitigate the effects of enteritis, attenuating the gut inflammatory response (Estensoro et al., 2016). Voorhees et al. (2019) demonstrated that 80% of FM replacement with bioprocessed soybean meal in rainbow trout diets affected neither fish growth performance nor intestinal health.

Rendered animal proteins

Among Aquafeed v2.0 class, rendered animal proteins represent another promising alternative protein source (Eroldoğan et al., 2023). These ingredients, derived from terrestrial livestock farms and slaughterhouse discards, include meals from blood, meat, bone, and feathers (Rasinger et al., 2016). However, the use of processed animal proteins (PAPs) was prohibited by the European Union (EU), after the transmissible spongiform encephalopathy (TSE) emergency (EC No 999/2001). The ban was later partially lifted, limiting the sources of these ingredients only to non-ruminant like swine- and poultry-derivatives (EC No 56/2013). PAPs are optimal substitutes for FM at appreciable levels, especially due to the absence on ANFs (Bandara, 2018). In fact, most products have a protein content comparable to, or even higher than FM, and they have good access to the global market (Hatlen et al., 2015). In addition, PAPs possess both high protein and lipid content, which make them very suitable for cost-effective aquafeed formulations. Finally, these ingredients are a natural source of several nutrients, such as amino acids (in particular lysine, sulfur amino acids, histidine and arginine) and phosphorus (Gasco et al., 2018).

Among animal by-products, blood meal is a cost-effective protein source, with significant quantities still being wasted in abattoirs across many countries (Hamed et al., 2017). Within fish diets, processing methods such as spray- and ring-drying are widely used for re-utilizing this source. Blood meal has received considerable interest in the aquafeed industry because of its high-quality protein content, excellent sources of lysine and histidine, and remarkable digestibility (ranging from 80-99%), along with the presence of different iron forms that contribute to the oxidation of feed components (Nogueira et al., 2012). Moreover, BM presents a reduced phosphorous content, which please fish farmers from both an environmental and industrial perspective (Agbebi et al., 2009).

Poultry by-products comprise ground poultry waste, including legs, necks, underdeveloped eggs, and a small amount of feathers (Bandara, 2018; W. Rossi & Davis, 2012). This rendered material, derived from slaughtered poultry discards, present protein level and amino acid profile relatively similar to FM, making the ingredient a valuable protein source (Hernández et al., 2014). Moreover, feather meal is rich in pepsin digestible protein (75-87%) and indispensable amino acids such as cystine, threonine, and arginine, making it a potential FM alternative (Arunlertaree & Moolthongnoi, 2008). Hu et al. (2008) evaluated that a high inclusion of poultry by-product meal in diets for gibel carp (*Carassius auratus gibelio*), can replace FM, without affecting growth and feed utilization. In a similar study, Sealey et al. (2011) showed that a complete replacement of FM by a diet based on poultry by-products supported growth, nutrient retention, and feed conversion ratios in rainbow trout.

Despite the promising qualities of rendered animal proteins, it should be pointed out that challenges emerge when included at high levels in fish diets. In fact, the percentage of inclusion, as well as the species considered, the deficiency of some EAAs and the low unsaturated fatty acids content essential for marine species, limit the use of PAPs within aquafeeds (Bandara, 2018; Gasco et al., 2018; Sealey et al., 2011). Additionally, the PAPs nutritional quality and nutrient availability highly depends on the raw material composition, freshness, and

processing technology applied (Gasco et al., 2018). For example, an increase in heat application has been reported to decrease lysine availability in blood meal, having significant effect on its digestibility (Bandara, 2018). From a nutritional perspective, feather meal is deficient in EAAs such as lysine, methionine, and histidine, and at higher inclusion levels have been shown to impair growth in several fish species (Bishop et al., 1995; Hasan et al., 1997). Moreover, processing aspects such as cooking time and pressure can affect the overall PAPs digestibility (Arunlertaree & Moolthongnoi, 2008).

Insects

In recent decades, efforts were focused on developing circular and regenerative ingredient sources, which can valorize by-products from other sectors or remediate nutrient discharges (Aquafeed v3.0) (Eroldoğan et al., 2023). Insects can be included in this category since they can bio-convert and bio-transform organic matter discharged from agri-food industries into products of high nutritional value (Mastoraki et al., 2022; Zarantoniello et al., 2020). Therefore, from a production standpoint, insect meal can be efficiently produced in farm facilities without requiring land or water resources for insects' cultivation.

A wide range of insect species are currently used in aquafeed production as alternative protein source. This includes housefly (*Musca domestica*), black soldier fly (*Hermetia illucens*), silkworm (*Bombyx mori*), yellow mealworm (*Tenebrio molitor*), lesser mealworm (*Alphitobius diaperinus*), house cricket (*Acheta domesticus*), banded cricket (*Gryllodes sigillatus*), and Jamaican field cricket (*Gryllus assimilis*), which are the most promising among others (Alfiko et al., 2022; Randazzo et al., 2021; Zarantoniello et al., 2023). Additionally, these species have been approved for aquafeed production under EU legislation (Commission Regulation (EU) 2017/893 of 24 May 2017). The suitability of insects within fish diets is due to their high protein content (35–61%) and favorable lipid content (13–33%), as well as other health-promoting factors, such as high energy, fat, and fiber content, minerals, vitamins, chitin, and other micro- and macro-nutrients (Bandara, 2018; Islam et al., 2024). Moreover, they contain high amounts of EAAs that make them suitable as protein source in substituting FM (Barroso et al., 2014; Henry et al., 2015). For instance, they present high contents of methionine, which is often a limiting EAA in animal- or plant-derived meals (Carvalho et al., 2023). Considering their potential, insect meals can also modulate fish immune system response, due to the presence of antioxidant and antimicrobial peptides, and chitin which stimulates the gut protective mucosal barrier, promoting animal health (Elieh Ali Komi et al., 2018; Henry et al., 2015; Stenberg et al., 2019). In fact, the exoskeleton of the insects is made of polysaccharide chitin, a polymer of glucosamine that, in low levels, can act as an immunostimulant (Henry et al., 2015; Maulu et al., 2022). However, chitin is generally considered as anti-nutritional factor, that fish cannot easily digest despite some species can produce chitinases enzyme (Henry et al., 2015; Lock et al., 2018). In fact, the chitin matrix may reduce the access of digestive enzymes to their substrates, reducing overall nutrient utilization and thereby growth (Belforti et al., 2016; Gasco et al., 2016). In terms of nutritional properties, both insect fatty- and amino acid-profile are affected by several factors, including taxon and species, their developmental stage, the rearing substrate and the manufacturing

processes of the subsequent meal (Barros-Cordeiro et al., 2014; Barroso et al., 2019; Hua et al., 2019; Islam et al., 2024). Compared to fish oil, the amounts of omega-3 poly-unsaturated fatty acids (i.e., docosahexaenoic acid, DHA; eicosapentaenoic acid, EPA) are generally lower in insect meals, reducing their suitability especially for marine species (Alfiko et al., 2022; Makkar et al., 2014). Moreover, insects contain large quantities of saturated fatty acids (SFA), which often impair both fish lipid composition and accumulation (e.g., liver steatosis) (Ewald et al., 2020; Zarantoniello et al., 2018). Nevertheless, for insect species such as *Hermetia illucens,* SFA are largely composed of lauric acid (12:0), which has been demonstrated to have beneficial effects on fish welfare due to its anti-inflammatory properties (Cardinaletti et al., 2019; Liland et al., 2017). Finally, dietary bioaccumulation of pesticides through insects has been also reported in fish (Ogunji et al., 2007).

Macroalgae and microbial biomasses (Single-cell proteins)

Aquafeed v3.0 comprises macroalgal and microbial sources which fully embrace the blue bioeconomy context of the aquaculture sector (Eroldoğan et al., 2023). These promising alternatives, primarily include seaweed, as well as microalgae, yeasts, bacteria, and fungi, which represent sustainable and renewable sources of protein-rich single-cell ingredients. These organisms can efficiently utilize nutrients from various waste streams and industrial by-products, while being environmentally friendly, with high productivity due to their short turnover time (Colombo & Turchini, 2021). The different classes of single cell-proteins (SCPs) can be dried and processed as a protein source, with specific nutritional profiles, or can be used alive as probiotics (Glencross et al., 2020).

Microalgae. Considering microalgae, there is a growing interest in their use as protein sources within aquafeeds. Microalgal resources are highly valued due to their environmental benefits and nutrient content such as proteins, phycobiliproteins/phycobilins, essential fatty acids, vitamins, and antioxidants (Eroldoğan et al., 2023; Glencross et al., 2020). Microalgal sustainability stems from the possibility of being cultivated on non-arable land, without using potable water, thereby minimizing water demand while not competing with traditional agricultural resources (Yarnold et al., 2019). Moreover, they can recover and convert nutrients from organic waste into high-quality feed ingredients, and offer the possibility of CO_2-uptake, providing the base for circular aquaculture industry (Venkata Mohan et al., 2016).

Besides their protein content, novel microalgal resources have received much attention due to their high content of essential PUFAs (i.e., genera *Nannochloropsis*, *Dunaliella*, *Chlorella*), especially beneficial for marine species (Shah et al., 2018). Among other compounds that microalgae can produce, astaxanthin and beta-carotene are particularly important (Eroldoğan et al., 2023). Astaxanthin, a red pigment, holds substantial commercial value as a food and flesh colorant, commonly used as an additive in fish feed (Lorenz & Cysewski, 2000). Beyond its role in enhancing flesh color, astaxanthin can also act as a precursor to vitamin A in certain fish species, while having antioxidant properties (Torrissen & Christiansen, 1995). The benefits of including

microalgae in the diet of cultured species encompass increased growth and survival rate, improved skin pigmentation, enhanced immunological responses, promoting overall health of the organisms (M. Jiang et al., 2019; Shah et al., 2018; Tibbetts et al., 2017).

Fungi. Biomass obtained from both terrestrial- and marine-fungi (e.g., yeast) has increasingly been investigated as potential aquafeed protein source, given its content in essential amino acids, PUFAs, fibers, minerals, and vitamins (Finnigan et al., 2017). Fungal biomasses or their derivatives can also be used as prebiotics, with beneficial effects demonstrated in several fish and shrimp species (Mohan et al., 2019; Sarlin & Philip, 2016). Moreover, marine fungi provide antimicrobial compounds that can be utilized in aquaculture against fish pathogens (Özkaya et al., 2017). In fact, fungal bioactive molecules exhibit a range of biological properties, including antioxidant, anti-cancer, antimicrobial, and immunostimulant effects. These molecules can enhance the fish innate immune system directly stimulating immune cells, or by promoting the growth of beneficial intestinal microbiota (Mohan et al., 2019).

Recent studies highlighted the environmental sustainability of mycoproteins compared to animal- or plant-based proteins, using life cycle assessment analyses (Mahboubi et al., 2017; Souza Filho et al., 2019). The advantages in producing mycoproteins for sustainable aquafeed formulations lie in their ability to grow in bioreactors with high metabolic efficiency, utilizing by-products such as carbon and nitrogen sources (Ahlborn et al., 2019; Rousta et al., 2021; Satari & Karimi, 2018). Additionally, yeast can be obtained as a by-product of brewery plants and agricultural waste fermentation process (Bandara, 2018).

Bacteria. Among SCPs, bacteria represent another promising protein source for aquafeeds. Besides vitamins, phospholipids and other functional compounds, bacteria can produce high values of crude proteins, essential amino acids, and bioactive secondary metabolites (Nasseri et al., 2011). Additionally, they can use various inexpensive and waste-based feedstocks, such as methane, methanol, synthetic gas, hydrogen, carbon dioxide, and sugars, as carbon and energy sources, to growth with minimum dependence on soil, water, and climatic conditions (Nasseri et al., 2011; Ritala et al., 2017).

Bacterial SCP-based products have shown to be effective growth promoters as alternative protein source, without having adverse effects on fish health, potentially replacing FM (Arnold et al., 2016; J. Wang et al., 2023). They can also improve immune response and survival rates in various aquaculture species (Hardy et al., 2018; Romarheim et al., 2011). Finally, live bacteria with probiotic effects can enhance host performance, by improving feed digestion and absorption, increasing diet nutritional value, and improving the quality of the rearing environment (Verschuere et al., 2000).

Macroalgae. Green (Chlorophyta), red (Rhodophyta), and brown (Ochrophyta) marine macroalgae are the main taxa of global seaweed biomass production (Moreira et al., 2022). Besides their valuable protein content, macroalgae extract present numerous compounds and secondary metabolites beneficial for farmed fish, owing to their prophylactic and therapeutic properties (Vatsos & Rebours, 2015). Moreover, all groups of seaweeds possess significant antimicrobial properties against many infectious agents, along with anti-inflammatory activities and immune modulation properties (Moreira et al., 2022; Vatsos & Rebours, 2015). The genera *Asparagopsis* (red seaweed) and *Sargassum* (brown seaweed) exhibit a broader range of antibacterial

properties, among others (Eroldoğan et al., 2023).

Despite the promising properties of SCPs, several limiting factors should be considered in their employment within aquafeeds. Considering microalgae, for instance, one of the primary challenges in their utilization is the nutrient composition variability among different strains. Differences in both their protein content and digestibility may be due to their complex cell wall structure, or to the presence of undigestible non-starch polysaccharides (Gong et al., 2018). Microalgae in fact possess a rigid and thick cell wall, mainly composed by polysaccharides and glycoproteins, that can affect nutrients digestion and absorption (Baudelet et al., 2017; Gong et al., 2018). Moreover, data on the substitution of FM by microalgae biomass indicate a limited level of substitution in which fish diets can be composed of microalgae derivatives (Eroldoğan et al., 2023).
During microbial biomasses production, the extraction method is one of the most important steps to consider, since it can affect the overall nutritional properties. In addition, large-scale production is currently extremely expensive, severely limiting the utilization of SCPs for farming purposes (Emblemsvåg et al., 2020; Hua et al., 2019). Indeed, with current technology, producing aquafeeds proteins from seaweed or microbial biomasses is neither economical nor environmentally sustainable without technical innovations to overcome the many existing gaps (Emblemsvåg et al., 2020).

Low palatability of alternative aquafeed ingredients in comparison to FM

Besides having an optimal amino acid profile that meets all the nutritional requirements of farmed species, FM presents a high- content of water-soluble substances such as free amino acids, small peptides, and nucleotides, which strongly stimulate fish feeding behavior (Al-Souti et al., 2019). Contrariwise, non-FM protein sources are generally lacking in these feed-stimulating substances, impairing the overall aquafeed palatability (Suresh et al., 2011).

Considering plant-based ingredients, besides having a lower content of water-soluble nitrogen-components, they present off-flavors and bitter substances that impair their organoleptic qualities within feeds. In fact, terrestrial plants often produce a number of secondary metabolites to ward off any threats in their natural environment (Bandara, 2018; Francis et al., 2001; Glencross, Baily, et al., 2020). These deterrents encompass biological compounds such as alkaloids, terpenes, saponins, and tannins, which present a bitter taste for fish (Francis et al., 2001). This results in a reduced acceptability and palatability of the vegetable meals by the farmed species, limiting their employment as feed ingredients owing to the difficult to remove these organic compounds.

Proteins of animal and plant origin are often subjected to hydrolysis during processing. This leads to the release of bitter-tasting peptides, which usually present hydrophobic residues in their structure (FitzGerald & O'Cuinn, 2006). Specifically, peptides <6 kDa with a high content of leucine proline, phenylalanine, tyrosine, isoleucine, and tryptophan are likely to be bitter, a negative aspect that is generally related to most protein hydrolysates. Among insect meals, the fatty acid profile (or the excess of lipid content), amino acid imbalances, presence of mycotoxins, fat rancidification, and the presence of antinutritional factors such as chitin, are important aspects that can affect their overall attractiveness within aquafeeds (Freccia et al., 2020; Islam et al., 2024).

With regard other above-described alternative protein sources, certain steps during their production processes may affect their attractiveness. For instance, increased heat applications usually deteriorates labile ingredients such as hemoglobin present in blood meal, reducing the palatability (Bandara, 2018). In yeasts, strains, storage, and production processes can affect the organoleptic qualities (Zheng et al., 2020). For seaweed, the chemical composition varies depending on environmental factors, including water temperature, salinity, light, nutrients and mineral availability, geographical distribution, and the seasonal cycle (Al-Souti et al., 2019). Finally, for those ingredients in which the poor palatability issues can be partially solved, it is not feasible to incorporate them at high levels to replace FM, or the large-scale production remains economically and environmentally disadvantageous.

A lack of palatability in aquafeeds has significant consequences for the aquaculture sector. A poorly palatable diet leads to scarce feed ingestion by fish, with severe implications for the environment and the farm's economy (Tacon & Metian, 2015). Above all, wasted feed represents a direct financial loss, also considering that aquafeeds account for about 50% of the total production costs (Ballester-Moltó et al., 2017; Beal et al., 2018; Ferrer Llagostera et al., 2019). Moreover, inadequate or inconsistent feeding can lead to poor fish growth performance, affecting the overall yield and profitability of the aquaculture operation (Elvy et al., 2022).

A low diet palatability forces fish farmers to overfeed, resulting in high levels of uneaten feed and fish feces that are released in wastewater, causing significant ecological damages (Ahmed & Thompson, 2019). In fact, the excess feed decomposes in the water release high concentrations of nutrients like nitrogen (N) and phosphorus (P), negatively affecting water quality and dissolved oxygen levels, and leading to eutrophication and harmful algal blooms (Ahmed & Thompson, 2019; Hall et al., 2018; White, 2013). Moreover, uneaten feed accumulates on the bottom of ponds or cages contributing to organic waste build-up and potentially creating anaerobic conditions that harm aquatic life (Dauda et al., 2019; Hall et al., 2018). The decrease in water quality can additionally cause disease outbreaks, affecting the health and welfare of the stock (Read & Fernandes, 2003). Finally, a reduced growth and health status affects the fish fillet quality, contributing to a reduced farming yields with significant economic impact (Gatlin et al., 2007).

1.2 Feed attractants

Several procedures are employed by aquafeed industries to achieve a better feed acceptability. Promoting nutrient consumption by farmed species is crucial to improve feed efficiency, which results in reducing feed losses while enhancing fish weight gain, thereby achieving better feed conversion ratios (FCRs). This leads to a reduction of carbon footprint, while safeguarding the economic investment, increasing the overall production yields.

Feed additives, which are included at low concentrations within fish diet, allow to achieve this goal using several strategies. These substances are in fact an essential part of the diet, and are utilized in aquaculture to improve the physical-chemical properties of the feed for different purposes: (i) mold inhibitors and antioxidants, are included to preserve the feed nutritional qualities; (ii) emulsifiers, stabilizers, binders, are included to facilitate both ingredients dispersion and pelleting; (iii) growth promoters, hormones, antibiotics, pre- and pro-biotics, are included to enhance the overall fish health conditions and growth; (iv) vitamins, amino acids, phospholipids, minerals, are added to supply essential nutrients; (v) feeding stimulants and feed colorants, included to enhance feed ingestion and consumer acceptance of the product (Hancz, 2020; Tacon, 1987).

Considering the latter category, feed attractants (FAs) are generally included in trace amounts in aquafeeds to enhance the feed attractiveness, stimulating fish feeding behavior by releasing water-soluble compounds (Hirt-Chabbert et al., 2012; Jeong et al., 2022). The chemicals released by FAs can activate the fish sensory districts such as taste and olfaction, initially stimulating olfactory receptors to encourage feed-seeking and ingestion (Kasumyan, 2019). This promotes quicker feed intake, minimizing the time that the feed remains in water and so preventing feed losses and deterioration of the water quality (Hirt-Chabbert et al., 2012; Shankar et al., 2008). However, along with the feed intake stimulation, FAs can also positively influence fish digestive processes, *via* the activation of taste receptors located at gut level, which trigger the release of key peptides and hormones (F. Li, 2013; Morais, 2017). This culminates in functional responses that regulate fish digestive and absorptive functions, secretion, intestinal motility, and metabolism (Morais, 2017). Finally, the use of FAs is essential in critical farming stages, such as the weaning of fish larvae from a living to a non-living diet, or during administration of medicated diet formulations, where the palatability aspect of the feed is fundamental (Oliveira & Cyrino, 2004).

Currently, the attractants included in aquafeeds to improve the palatability are mostly represented by marine extracts (Suresh et al., 2011). Meals derived from mollusks (e.g., squids, mussels, clams), crustaceans (e.g., shrimps), marine polychaetae worms, and forage fish (e.g., fish solubles and fish protein hydrolysates), are generally recognized as potent attractors for several aquatic species (Hancz, 2020).

In this regard, several studies investigated the potential of these marine-derived ingredients for their attractive properties. For instance, Nagel et al. (2014) evaluated the application of blue mussel (*Mytilus edulis*) meal at different inclusion levels in a plant-based diet for turbot (*Psetta maxima*), concluding that its addition resulted in an increased daily feed intake and growth performance for the fish. In a similar study, Kader et al. (2012)

tested the effects of fish soluble (*Katsuwonus pelamis* by-product), krill meal, squid meal, and crystalline amino acids in a dehulled soybean meal diet for red sea bream (*Pagrus major*), demonstrating that all the tested ingredients positively stimulated the fish feeding behavior. Ho et al. (2014) tested the potential of Pacific hake (*Merluccius productus*) hydrolysates on juvenile chinook salmon (*Oncorhynchus tshawytscha*), demonstrating its potential as a dietary attractant for salmonid plant-based diets.

The manufacturing process of marine by-products generally consists in their preservation in formic acid as silage, and in their subsequent conversion into fish protein concentrates, finally obtaining the attractants meals (Utne-Palm et al., 2020). Squid industry by-products, for instance, can be converted into hydrolysates intended as attractants within aquafeeds (Pan et al., 2022).

The primary negative aspect of these ingredients, however, is their marine origin, which pose significant unsustainability concerns for the sector (Ytrestøyl et al., 2015). In fact, whilst the current alternative protein sources aim to completely replace the FM levels, the feed attractant's sector still relies on natural-derived ingredients to a large extent. In addition, the different processing methods, as well as the natural origin, play a crucial role in determining the effectiveness of these substances. Their diverse composition and availability, which depend on their harvesting location, along with the quality and freshness, influence their overall attractiveness, resulting in a limited reliability when included in aquafeeds (Bureau et al., 2000; Goda et al., 2007). This ultimately leads to the inability to standardize the FAs attractive effect, which therefore exhibits highly variable outcomes.

Marine extracts are a good source of a limited number of organic molecules, which strongly stimulate fish appetite (Kim & Cho, 2019). Specific compounds such as free amino acids (e.g., glycine, L-alanine), small chain peptides, nucleosides, nucleotides, betaine, and taurine, are well-recognized potent attractants for fish and shrimp (Di Peng et al., 2022; Suresh et al., 2011; Tusche et al., 2011). In this regard, several solutions have been explored to evaluate the effectiveness of these molecules within aquafeeds. For instance, Takakuwa et al. (2019) explored how jack mackerel muscle extract influences the feeding behavior of greater amberjack (*Seriola dumerili*), concluding that nucleosides within the extract triggered the most pronounced feeding response. Similarly, in a study conducted by Olsén & Lundh (2016), the effects of five L-amino acids (alanine, arginine, glycine, leucine, and serine) on crucian carp (*Carassius carassius*) provided comparable outcomes. Yue et al. (2022) evaluated the effects of limonene, allicin, and betaine supplementation in a low FM diet for largemouth bass (*Micropterus salmoides*), highlighting that at 0.2% concentration they could improve the fish zootechnical performance.

Among the different attractive molecules explored, amino acids were found to exert the greatest attractive effect in farmed species (Li et al., 2022). However, their inclusion within aquafeeds present several limitations and disadvantages: (i) their attractive effects greatly varies across fish species and life stages considered, with insufficient data on optimal dosages in available scientific literature; (ii) their concentration and interaction with other components in the feed can affect their overall attractive performance; (iii) the attractiveness of each type of amino acid is strictly species-specific, emphasizing the need for tailored approaches in aquafeed formulation; (iv) the effectiveness of amino acids in attracting fish is generally lower compared to the above-

mentioned marine-derived extracts (X. Li et al., 2022; Newton et al., 2023; Tusche et al., 2011). Hence, the choice and concentration of amino acids can significantly impact feed efficiency, highlighting the need for further investigation in this area to obtain an optimal feeding response by the farmed species (X. Li et al., 2022; Tusche et al., 2011). More specifically, it is becoming crucial to search for a more viable and sustainable solution, that provides a standardized and reliable attractive effect in improving the palatability of aquaculture diets.

1.3 The influence of nutrition on fish physiology

Any physiological responses and/or behavioral changes in organisms involve biological reactions mediated by cellular molecular mechanisms (Janz, 2013). In this context, molecular biomarkers directly reflect gene activity and could serve as reliable early indicators across various pathways, including those involved in feeding. In fact, both the regulation of nutritional status and energy balance underpins homeostatic mechanisms which drive the foraging and consumption of feed.

In fish, taste and olfaction play a central role in driving feeding behaviors (Valentinčič, 2004). These sensory systems allow to discriminate a broad range of compounds found in the aquatic environment, permitting to determine their harmful or nutritionally relevant origin (Kasumyan, 2019). Chemoreception is thereby a primary mechanism for fish to distinguish and select specific odorants and locate their source (Derby & Sorensen, 2008). Considering this aspect, promoting feed chemical attraction is of crucial importance in aquaculture to stimulate fish feeding behavior and elicit an optimal feeding response. By administering highly palatable feeds, which release water-soluble attractive compounds, the fish appetite can be effectively stimulated, enhancing feed seeking and ingestion (Hancz, 2020; Morais, 2017).

Homeostatic appetite regulation

Despite the enormous variability of habitats and ecological niches occupied by over 30,000 species, teleost exhibit a highly conserved control system of appetite (Volkoff, 2016). In general, the reception of aquatic signals by the sensory systems can trigger endocrine changes in the organism, activating several mechanisms. Feed-stimuli from external environment are integrated with endogenous factors, particularly at the brain-central level, triggering biochemical responses through neurohormonal signals (Volkoff, 2016). More specifically, the elaboration of these feedback occurs *via* an important cross-talk between the brain, at central level, and peripheral organs such as the gastro-intestinal tract (Rønnestad et al., 2017). Starting from the hypothalamus, these signals are integrated by peripheral chemicals (e.g. glucose) or endocrine factors (e.g. gastrointestinal hormones) that have a direct action on feeding centers (Lin et al., 2000; Volkoff et al., 2005; Kulczykowska & Sánchez Vázquez, 2010; Volkoff et al., 2009; Volkoff & Peter, 2006). The primary role of these stimuli is to maintain overall homeostasis by monitoring and regulating the body's energy balance.

Orexigenic (appetite-stimulating) and anorexigenic (appetite-inhibiting) signals are both involved in these pathways. Among the major appetite-stimulating factors found at the brain/central level, neuropeptide Y (along with NPY/agouti-related peptide -AgRP- neurons) is the most potent orexigenic signal (Bertucci et al., 2019). Neuropeptide Y is a 36kDa protein, belongs to the NPY family of peptides, and it is mainly secreted by the neurosecretory cells at hypothalamus level. Several studies showed that in a number of fish species, including zebrafish (*Danio rerio*) (Tian et al., 2015), goldfish (*Carassius auratus*) (Narnaware & Peter, 2001), and chinook and coho salmon (*Oncorhynchus tshawytscha*; *Oncorhynchus kisutch*) (Silverstein et al., 1998), feed deprivation increased *npy* brain expression, suggesting its appetite-stimulating role in fish.

Additional orexigenic factors, involved in the central control of appetite, have been reported in brain teleost. Among these, orexin and galanin are appetite-stimulating neuropeptides with a potent orexigenic role (Rønnestad et al., 2017). Orexin has been shown to stimulate feeding and locomotor activities in both mammals (Tsujino & Sakurai, 2009) and fish (Facciolo et al., 2011; Penney & Volkoff, 2014), and its brain-mRNA expression usually increase following periods of fasting (Buckley et al., 2010; Nakamachi et al., 2006; Novak et al., 2005; M. Xu & Volkoff, 2007). Galanin (GAL), instead, is a 29-30 amino acid peptide that acts in the brain and peripheral tissues to enhance appetite and to regulate metabolism (Rønnestad et al., 2017).

Central appetite signals are fine-regulated by the action of endocrine signals produced by peripheral organs, and particularly by the gastro-intestinal tract (Furness, 2016; Murphy & Bloom, 2004; Rønnestad et al., 2017). In fact, appetite-regulating factors from the gut are involved in digestive processes, thus coordinating both hunger and satiety signals. The gut-exocrine cells activity is influenced by the presence, transition, and digestion of nutrients, which stimulates or inhibits the secretion of different peptides (Rønnestad et al., 2017). In this context, ghrelin (GHRL), a 28 amino acid peptide mainly produced in the stomach of fish, or in the gut of stomachless species, is the most important peripheral orexigenic signal, and its secretion stimulates the central/brain expression of NPY (Gorissen et al., 2006).

Along with the appetite stimulation/hunger sensation, a *plethora* of anorexigenic signals counteract the action of the above-described stimuli, inducing a satiety sensation (Rønnestad et al., 2017; Volkoff, 2016). The hormone leptin, a 16kDa protein primarily secreted by the liver, plays a key role in informing the brain about the nutritional status and energy reserves of the organism (Bertucci et al., 2019; Gorissen et al., 2006). In fact, leptin receptors are present on both NPY+/AgRP+- and POMC+/CART+- neurons, and influence feed intake by simultaneously inhibiting orexigenic while stimulating anorexigenic factors. More importantly, leptin is secreted into the bloodstream in proportion to the total amount of body fat and is therefore a reliable indicator of energy reserves availability (Niswender et al., 2004; Schwartz et al., 2000). In fact, lipid accumulation in the fish body typically increases in proportion to the amount of feed ingested, as a result of higher energy availability that can be stored (Oswal & Yeo, 2010). For instance, a scarce feed efficiency also tended to have low lipid retention, as demonstrated in both European sea bass (*Dicentrarchus labrax*) (Peres & Oliva-Teles, 1999) and grass carp (*Ctenopharyngodon idella*) (DU et al., 2005).

Along with the leptin action, several anorexigenic signals are secreted by central and peripheral organs. For instance, the cocaine- and amphetamine-related transcript (CART) peptide, and the melanocortin system (including pro-opiomelancortin -Pomc- prohormone), both expressed in the brain, as well as the gut-expressed cholecystokinin (CCK), mainly act as short-term satiety factors (Rønnestad et al., 2017; Volkoff, 2016).

Monoaminergic systems

In teleost, the regulation of appetite involves both homeostatic mechanisms and hedonic/motivational neurocircuits, which are mediated by monoaminergic systems (de Pedro & Björnsson, 2001; M. A. Rossi & Stuber, 2018). In fact, feeding is primarily influenced by the organism's energy status, which depends on the nutrient availability in the environment. However, the feed's organoleptic qualities can motivate feeding exerting a powerful effect on its regulation at central level (Erlanson-Albertsson, 2005). Ingesting a highly palatable feed can overlaps the appetite-regulating endocrine pathways, motivating feeding by inducing pleasure and reward sensations despite hunger or satiety (Pelchat, 2002; M. A. Rossi & Stuber, 2018; G.-J. Wang et al., 2004). For example, diets with high fat or sugar content, as well as added with artificial sweeteners, have strong attractiveness either because they can be quickly converted into energy, or because they can stimulate the brain independently of their caloric value (Morais, 2017; Nesse & Berridge, 1997; Onaolapo & Onaolapo, 2018; Shirazi-Beechey et al., 2014; G.-J. Wang et al., 2004).

Among the monoaminergic systems, the mesolimbic dopamine system is directly involved in the regulation of feeding behavior. In fact, dopamine (DA) is the main neurotransmitter involved in the reward pathway, and both dopamine levels and dopaminergic transmission (receptors-mediated) change in response to appetitive stimuli (De Macedo et al., 2016). In vertebrates, dopamine receptors are classified into two distinct families known as D1-like and D2-like receptors (K. Yamamoto & Vernier, 2011). These families represent two separate classes of receptors, each with unique structures and functions, which are intricately linked to feed intake regulation (Alsiö et al., 2010; Le Crom et al., 2003). In previous studies, a long-term exposure of palatable diet in rats promoted feeding behavior, influencing the expression of dopamine D2 receptors (Baik, 2013; P. M. Johnson & Kenny, 2010). In addition, transgenic mice, unable to synthesize dopamine, died of starvation due to a lack of motivation to eat (Szczypka et al., 1999). Similarly, in fish, dopamine treatment induces an increase in locomotor and seeking activities (Mok & Munro, 1998). For example, oral L-dopa administration, a precursor of dopamine, inhibited European sea bass food intake and growth (Leal et al., 2013). Overall, fasting appears to stimulate the central synthesis of monoamine pathways, increasing their levels in the brain. This is translated into modifications in hedonic feeding, or desire to eat, motivating fish to seek feed (Mandic & Volkoff, 2018). However, despite some findings, these mechanisms are still poorly investigated in fish.

Among monoamines, serotonin (or 5-hydroxytryptamine, 5-HT) is another neurotransmitter that has been shown to influence feeding in vertebrates (Mandic & Volkoff, 2018). Particularly, the stimulation of serotonergic system is generally related to a decrease in feed ingestion, suggesting an anorexigenic role for this neurotransmitter. In teleost, different subtypes of serotonin receptors are reported. For instance, in rainbow trout, the anorexigenic action of 5-HT is probably mediated by activation of 5-HT1A and 5-H2C-like receptors (Pérez Maceira et al., 2014). In goldfish (*Carassius auratus*), the stimulation of serotonin release and the suppression of its reuptake, using Fluoxetine, inhibited fish feed ingestion (Mennigen et al., 2009).

Growth

A detailed understanding of how nutrients are perceived by the organism and influence growth mechanisms, becomes crucial for the aquaculture industry to achieve high production yields (Canosa & Bertucci, 2020). Designing better and more efficient formulated diets can enhance growth and welfare, which are important aspects for cultured aquatic species. In this regard, stimulating feeding can increase the supply of nutrients to the body, which can be more invested in different physiological mechanisms by fish.

In the somatic growth process, nutrients are converted by metabolic reactions into cell and tissue components. The endocrine system that regulates this mechanism is referred to as the somatotropic axis (Canosa et al., 2007). This includes: (i) the hypothalamus and its hypophysiotropic factors; (ii) the pituitary gland and the growth hormone (GH) (a peptide primarily synthesized and released by the somatotropic cells of the anterior pituitary gland); (iii) the liver, as well as peripheral tissues, and the insulin-like growth factors (IGFs) (all displaying a key role in regulating body size) (Chang & Wong, 2009; Gahete et al., 2009; Yada, 2007). Insulin-like growth factor 1 (IGF1) acts as a downstream effector of growth hormone (GH) signaling in vertebrate, collectively constituting the somatotropic (GH/IGF1) axis (Nornberg et al., 2016). This represents one of the most important endocrine systems that regulate skeletal and soft tissue development, as well as the growth-related processes (Triantaphyllopoulos et al., 2020). In some teleost fish, IGF1 expression in blood or other tissues appears to reflect the fish's nutritional state, showing a positive correlation with dietary ratio, nutrient content, and overall body growth rate (Ndandala et al., 2022). Opposing to this pathway, myostatin (MSTN), also called growth and differentiation factor 8, has been shown to be a negative regulator of satellite cells growth and postnatal myogenesis (Gao et al., 2016). As a member of the transforming growth factor β (TGFβ) superfamily, myostatin is typically expressed in skeletal muscle, exerting growth-inhibitory effects (Kerr et al., 2005).

This extensive knowledge about the fish growth mechanisms can be applied in aquaculture research to develop nutrient-based strategies, for maximizing the production of cultured species and evaluating the suitability of tested ingredients. Understanding how dietary macronutrients affect fish growth is of great value, as it allows for changes in diet composition, to ensure high growth performance while reducing feed costs, optimizing overall aquaculture production.

Reproduction

Fish often modulate their reproductive physiology based on their available energy reserves, since they frequently face periods of reduced nutrient availability during their natural life cycle (Volkoff & London, 2018). When malnourished conditions occur, fish encounter reproductive issues, including failures in completing vitellogenesis, oocyte maturation, and egg laying in females (Volkoff & London, 2018; Yilmaz et al., 2018), as well as impaired concentration, motility, viability, morphology, and fertility of spermatozoa in males (Lombó et al., 2024; Pamungkas et al., 2023). For that reason, depending on environmental changes, nutritional status, and body growth rate, the fish endocrine system regulates internal responses, for example,

by inhibiting reproductive pathways to preserve energy supplies for vital functions (Izquierdo et al., 2001; Weltzien et al., 2004). In fish, a number of peptides including NPY and orexin, alongside peripheral factors like leptin and ghrelin, have been identified to regulate these mechanisms (Volkoff & London, 2018). In fact, the nutritional status is crucial to sustain the high-energy demands for gametes production and reproductive behaviors, evidencing a clear relationship between feeding and reproduction. For that reason, especially in controlled aquaculture systems, feed formulation is one of the few variables becoming a key factor in achieving these outcomes.

The fish reproduction cycle involves several stages which culminate in the fertilization of eggs by spermatozoa to produce offspring. Similar to other vertebrates, in fish both the production and release of gametes are under hormonal control (Weltzien et al., 2004). Particularly, reproduction is regulated by the hypothalamus-pituitary-gonadal (HPG) axis, that involves the hypothalamic gonadotropin-releasing hormone (GnRH) (Volkoff & London, 2018). GnRH regulates the synthesis and release of pituitary gonadotropins, including follicle-stimulating hormone (FSH) and luteinizing hormone (LH). The gonads' developmental stage is under the control of FSH while their maturation phase primarily occurs under the influence of LH (Clelland & Peng, 2009; Khan & Thomas, 1999; Patiño et al., 2001). Follicle-stimulating hormone is secreted into the bloodstream by the pituitary gland and acts on follicle cells which surround developing oocytes to induce synthesis of the sex steroid hormone, estradiol-17β (Hara et al., 2016). This estrogen induces vitellogenesis in the hepatocytes, acting *via* endocrine regulation eventually stimulating vitellogenin gene expression.

During this process, females invest considerable energy to supply the eggs with nutritional reserves (i.e. yolk/vitellogenin) (Clelland & Peng, 2009; McBride et al., 2015). Yolk proteins are first synthesized by maternal hepatocytes and then transferred to the growing oocytes through the bloodstream (Hara et al., 2016). Among the yolk proteins, vitellogenin is a high-molecular-mass complex protein consisting of carbohydrates, lipids, phosphorus, and binding other elements such as calcium, iron and zinc (Finn, 2007; Smolenaars et al., 2007). Its uptake into oocytes is the main mechanism for their development, and thus Vtg gene expression is a reliable molecular marker for assessing broodstock reproduction (Brooks et al., 1997). In fact, the production of fertilized eggs with high potential hatching rates, capable of developing into commercially viable sizes, is crucial for the aquaculture industry. Besides producing large quantities of eggs, it is required a high egg quality to support normal development and ensure high survival rates of offspring to mature stages (Lubzens et al., 2017).

Gonads examination, measurement of sex steroid levels, and gonadal indices are further effective methods for assessing fish reproductive status (Volkoff & London, 2018). The gonadosomatic index (GSI), which compares the relative size of the gonads with total body mass is commonly used as a reproductive stage indicator (Wagle, 2015). GSI increases as the fish approaches maturity, reaching its highest point during peak reproductive readiness. Additionally, fecundity, which measures the number of eggs produced by a fish either per spawn or relative to its body weight, is also used to evaluate the reproductive potential of female fish.

In fish, as in all vertebrates, puberty is reached when individuals attain the appropriate age and size, having gathered sufficient energy reserves to satisfy the high-energy demands of maturation (Okuzawa, 2002).

In many species, decreasing the feeding rates of mature fish during the reproductive cycle results in reduced growth and gonadosomatic index (GSI), delayed gonadal maturation, and diminished spawning frequency and duration (Volkoff & London, 2018). For example, in a study conducted on immature coho salmon females, a prolonged fasting impaired endocrine function by reducing growth-related hormones (IGF-1) (Y. Yamamoto et al., 2011).

Immune and stress responses

In fish, the gastrointestinal tract plays a crucial role in nutrient digestion and absorption, as well as in immune responses, while liver is pivotal in many pathways, including metabolism, detoxification, and nutrient storage (Carnevali et al., 2017; Urán et al., 2008; Z. Zhou et al., 2018). Malnutrition is a recognized stressor, making it essential to evaluate both morphological structure and macromolecular composition of both these organs in response to new aquafeed formulations to ensure optimal fish rearing conditions (Piccinetti et al., 2012). An adequate nutrient supply to the organism is strictly dependent on the correct functionality of liver and intestine, which should be deeply analyzed when administering new dietary formulations. At the same time, a well-designed diet should promote the overall physiological responses of such organs, without having adverse effects on their functionality.

Among the various physiological functions of the gut, immune defense is one of the most important, being a physical barrier to pathogen entry, mediated by the gut-associated lymphoid tissue (GALT) (Salinas & Parra, 2015). In fact, the fish mucosa-associated lymphoid tissues (MALT), which comprises mucosal surfaces such as skin, gut, gills, and olfactory organ, serve as crucial first lines of defense against pathogens and environmental threats (Islam et al., 2024). The exposure of food antigens and anti-nutritional factors, as well as bacteria, viruses, parasites, and toxins, continuously challenge the gastrointestinal tract (Lazado & Caipang, 2014; Salinas & Parra, 2015). When intestinal epithelial cells get infected or damaged, they release several molecules involved in innate immunity, acting as danger signals that prompt the immune system to respond and control the infection (Gomez et al., 2013). Through pattern recognition receptors (e.g., TLRs and NOD receptors) are triggered by signaling cascades that lead to the initiation of mucosal inflammation (i.e., enteritis). These involves the expression of transcription factors (i.e., NFk-B), cytokines (i.e., interleukins and interferons), and chemical mediators (i.e., prostaglandins and nitric oxide) (Salinas & Parra, 2015). At the morphological level, these responses result in visible changes which include shortening of intestinal villi, loss of enterocyte supranuclear vacuolization, enlargement in the lamina propria of the mucosal folds, and infiltration of inflammatory cells into the lamina propria (Raskovic et al., 2011). For this reason, these signals, combined with a tissue morphological evaluation, are important biomarkers useful in highlighting possible pathological states of the gastro-intestinal tract.

In this regard, if the nutrient absorption by the intestine is not adequate, or the diet formulation is not suitable, histological changes in the liver can be easily recognized. Alteration of liver parenchyma, such as hepatocytes

vacuolization, liver fatty degeneration, changes in metabolic activity, and necrosis are the most common observed (Raskovic et al., 2011). Liver morphometric parameters are commonly used to indicate the metabolic activity of hepatocytes, including the hepatocytes number, as well as both their surface and nuclear area, as well as the glycogen and lipid content in the cytoplasm.

Despite the above-mentioned evaluations, the assessment of stress status requires the investigation of multiple biomarkers to obtain a comprehensive evaluation. For example, behavioral and physiological responses to stress serve as prevailing indicators of impaired well-being (Ashley, 2007; Segner et al., 2012).
In teleost, cortisol predominates among corticosteroids, exerting a significant influence in various physiological responses such as growth, immunoregulation, and energy maintenance (Tsalafouta et al., 2018). As a consequence, the activation of stress response increases cortisol levels, triggering several reactions including alterations in metabolism, osmoregulation, and immunosuppression (Schreck et al., 2016). Two physiological axes are activated in a cooperative manner during stress situations: the Hypothalamus-Pituitary-Interrenal axis (HPI), where corticosteroids are produced by the head kidney, and the Hypothalamus-Sympathetic-Chromaffin axis (HSC), where catecholamines, like epinephrine and norepinephrine, are produced by chromaffin cells (Khansari et al., 2019; Rotllant et al., 2006). The HPI axis involves the immediate secretion of corticosteroid-releasing hormone (CRH) by the hypothalamus, which subsequently stimulates the release of adrenocorticotropic hormone (ACTH) into the bloodstream by the pituitary gland (Parra et al., 2015). ACTH then stimulates the release of cortisol from the interrenal cells of the head kidney, as well as of melanocyte-stimulating hormone (α-MSH) (Tort, 2011). The cortisol release increases neutrophils, reduces lymphocyte counts and antibody production, and leads to a reduction in cytokines levels such as TNF-α, TGF-β, and IL-6, having significant effects on immune responses (Parra et al., 2015). The physiological effects of cortisol are exerted by the interaction with the mineralocorticoid receptor (MR), and both glucocorticoid receptors 1 (GR1) and 2 (GR2) (Vallejos-Vidal et al., 2022). These receptors are differently influenced by cortisol levels, with GR1 recognizing cortisol at higher concentrations, while GR2 responds to very low concentrations (Vazzana et al., 2010).

The health and welfare of farmed fish are of paramount importance in farming practices, and the use of tailored diets has become essential to ensure this aspect. A scarce nutrient ingestion primarily influences fish zootechnical performance, as a consequence of impaired well-being, and this should be considered in aquaculture-related research to establish the nutritional requirements of the different species (Canosa & Bertucci, 2020; Hack et al., 2018). Thereby, besides designing a fine-tuned diet formulation, it is crucial to ensure its proper ingestion. For this reason, in a sustainable development of the aquafeed sector, which relies on viable but low palatable ingredients, the use of reliable and eco-friendly substances to increase palatability is mandatory.

1.4 Aim of the thesis

The aquafeed sector is currently one of the main limiting factors of an ever-expanding aquaculture industry (Beal et al., 2018; Ye et al., 2020). The unsustainability concerns of the traditional formulated diets, which still relied on marine-derived protein sources (i.e., FM), promoted research activities towards more sustainable ingredients (Eroldoğan et al., 2023). Thereby, in recent decades, efforts have been focused on including more sustainable protein sources in fish diets, mainly consisting of plant-derived meals, followed by terrestrial livestock by-products, insects, algae, and microbial sources. However, although these ingredients may meet the nutritional requirements of the target species, their often-poor palatability still remains one of the major challenges for the aquaculture sector (Suresh et al., 2011).

A lack of palatability in aquafeeds has significant consequences, primarily leading to scarce feed ingestion by fish. This has negative implications for the farm's economy and the environment since wasted feed represents a direct financial loss which affects water quality, threatening aquatic life and the entire ecosystem (Ahmed & Thompson, 2019; Ballester-Moltó et al., 2017; Tacon & Metian, 2015). Additionally, inadequate or inconsistent feeding can result in suboptimal fish growth, negatively impacting the overall yield and profitability of the aquaculture operation (Elvy et al., 2022).

Nowadays, natural-derived FAs are commonly used within aquafeeds to enhance feeding response by the fish (Hancz, 2020). However, these substances are mainly represented by marine extracts, derived from mollusks, crustaceans, marine polychaetae worms, and forage fish, posing further unsustainability issues (Hancz, 2020; Ytrestøyl et al., 2015). Moreover, their harvesting location, composition, freshness, and processing methods influence their overall attractiveness, which is highly variable (Bureau et al., 2000; Goda et al., 2007).

The alternative solutions that are currently used in substitution to FAs are represented by substances such as free amino acids, nucleotides, nucleosides, small chain peptides, betaine, and taurine (Di Peng et al., 2022; Suresh et al., 2011; Tusche et al., 2011). Among these compounds, amino acids are potent attractors for fish, presenting, however, limitations and disadvantages in their use since their attractiveness varies depending on fish species and life cycle considered, as well as on concentration and interaction with other molecules present in the feed (X. Li et al., 2022; Tusche et al., 2011). Finally, the attractive effect of amino acids is generally less strong compared to marine extracts (X. Li et al., 2022; Newton et al., 2023).

Considering these aspects, synthetic flavors produced through fine-tuned methods could serve as a novel, standardized, and sustainable alternative to natural feed attractants. The existing literature includes only a few specific studies on this topic (Hancz, 2020), suggesting a significant opportunity for the aquaculture sector to meet the organoleptic requirements of fish (Reig et al., 2003). In the food industry, it is well-established that a limited number of molecules can replicate specific flavors (Januszewska et al., 2020). Synthetic flavors generally consist of a blend of aromatic chemicals, such as esters, terpenes, aldehydes, phenols, lactones, fatty acids, and sulfur compounds (Singh et al., 2023; A. M. Ulloa, 2018), all deemed safe by the American Flavor and Extracts Manufacturers Association (FEMA). These substances are mixed in a liquid solvent (e.g., water,

triacetin, propylene glycol) to enhance or create specific flavors (e.g., banana, cherry, chocolate) in various products (Reig et al., 2003; A. M. Ulloa, 2018). This approach allows for the sustainable and cost-effective faithful reproduction of thousands of food-related flavors (Torres et al., 2010).

In this context, the aim of the present thesis was to identify different liquid synthetic flavors, already widely used for human purposes, and test them firstly on an experimental model (zebrafish; *Danio rerio*), considering its whole life cycle, and then focusing on two commercially relevant marine species for the Mediterranean aquaculture (European seabass, *Dicentrarchus labrax*; gilthead sea bream, *Sparus aurata*), to assess their potential role as feed attractants.
A number of laboratory analyses have been performed, including behavioral tests, biometric measurements, histological, molecular, and immunohistochemical investigations, giving emphasis on feed intake, zootechnical performance, overall welfare and reward mechanisms of fish, using a multidisciplinary approach.

In the first section (Chapter 2), a number of positive/attractive and negative/repulsive commercial synthetic flavors were initially identified through a behavioral preference test performed on zebrafish larvae. Subsequently, three selected flavors (cheese and caramel – attractive; coconut – repulsive), as well as the solvent used for their production (propylene Glycol; PG), were tested during a two-month feeding trial on zebrafish early development (from larvae to juveniles), to assess their potential role as feed attractants. Using a multidisciplinary approach, a comprehensive overview of the fish physiological responses was evaluated.

In the second section (Chapter 3), the same synthetic flavors (cheese, caramel, and coconut) and PG tested in the first section, were added to a specific zebrafish diet, provided from fish larvae to adults. The aim of this second section was to assess the potential role as feed attractants during the whole fish life cycle (six months), giving emphasis on fish intake, growth, welfare, feed reward mechanisms, and reproductive performances.

The results previously obtained on zebrafish model (an omnivorous species) have been a crucial starting point for further investigations in farmed fish species. Thereby, in the third section (Chapter 4), the effectiveness of cheese-, caramel-, and coconut-synthetic flavors, as well as PG, were tested on European seabass (*Dicentrarchus labrax*), during a 90-day feeding trial, evaluating the fish physiological responses. This marine carnivorous species is of significant importance especially for the Mediterranean aquaculture, presenting a high market value (Vandeputte et al., 2019). Thereby, up to this phase, the trials allowed to have a comprehensive overview of synthetic flavors as feed attractants in both freshwater and marine species, which present different feeding behaviors.

Finally, based on the results obtained in the previous feeding trials, the last section (Chapter 5) proposed the application of a novel technology to implement the inclusion method of synthetic flavors within aquafeeds. In fact, during this phase, both cheese and caramel synthetic flavors have been subjected to a micro-encapsulation process (Aragão et al., 2014; Willer & Aldridge, 2019). This allowed to include the liquid flavors in the wall of natural-based microcapsules, which was able to stick to any aquafeed and quickly release the flavors once in contact with water, while preventing their leaching (Bonilla-Ahumada et al., 2018; Mahotra et al., 2022).

After the implementation process, both micro-encapsulated cheese and caramel flavors were added to a diet intended for gilthead sea bream (*Sparus aurata*), during a three-month feeding trial, evaluating the fish physiological responses. Moreover, since the previous feeding trials (described in first three sections – Chapters 2, 3 and 4) were conducted under laboratory rearing conditions, this last trial was conducted in open circulation tanks, with similar rearing conditions to those found in fish farms. In fact, gilthead sea bream is another important species in Mediterranean aquaculture, along with European seabass (FAO, 2022).

1.5 Model organisms

Zebrafish



Class: Actinopterygii
Order: Cypriniformes
Family: Cyprinidae
Genus: *Danio*
Species: *Danio rerio*

Zebrafish (*Danio rerio*) is a tropical/subtropical cyprinid fish with origins in South Asia, ranging from Pakistan, India, Bangladesh, Nepal, and Myanmar, and evolved around 320 million years ago (Lawrence, 2007; Ribas & Piferrer, 2014). It is a freshwater species that adapted to a variety of different habitats including rivers, small streams, channels, stagnant or slow-moving pools, such as heels and rice paddies (Spence et al., 2006; Varga, 2016). Zebrafish is considered a eurythermal species, that tolerates temperatures ranging from 16.5 to 38.6 °C, in the wild (López-Olmeda & Sánchez-Vázquez, 2011). Additionally, it can be found in aquatic environments with different acidities, with pH values ranging from 5.5 to 9 and also with various amounts of dissolved salts (Engeszer et al., 2007; McClure et al., 2006).

The diversity of climates and natural habitats, with considerable variations in their physic-chemical characteristics, along with the notable resilience and adaptability of zebrafish, allows for various breeding methods that can be tailored to different research objectives (Lawrence, 2007). In fact, the physic-chemical properties of the water under laboratory conditions may differ from those found in the wild. Zebrafish is typically reared under controlled conditions, with standard rearing temperatures narrowed at 26 –29 °C and preferably set at 28 °C, and with pH of 7–8, CaCO3 75–200 mg L^{-1}, and conductivity 700–1000 lS (Detrich et al. 2004; Lawrence, 2007; Westerfield 1995).

With regard feeding behaviors, zebrafish is considered euryphagous omnivore (P. E. Ulloa et al., 2011). In the wild, adult zebrafish primarily feeds on zooplankton and insects, but phytoplankton, filamentous algae, spores, invertebrates, and arachnids may also be included (Spence et al., 2008).

Despite these behaviors, each stage of the zebrafish life cycle requires the appropriate type of food. Current feeding practices involve diets based on formulated microparticles, integrated with ciliates, rotifers, and Artemia nauplii (Westerfield 1995; Best et al., 2010). More specifically, considering larval stage, which is a critical phase during fish rearing, feed has to be provided several times a day, especially between 6 and 15 days post-fertilization (dpf), as during this period the demand is constant. During larval stage, feed supply is initially based on rotifers (*Brachionus plicatilis*) and subsequently on *Artemia salina*, both integrated with powdered dried feed (Best et al., 2010; Lawrence, Sanders, et al., 2012). Subsequently, from 15 to 40 dpf (metamorphic and juvenile stages), feed supply is mostly based on dried feed supplemented with artemia to 3–5 takes a day. In adulthood, fish feeding is mainly based on dried feed, with Artemia supplement from 1 to 3

times a day (Lawrence, 2011; Lawrence, Best, et al., 2012). Adult zebrafish mainly feed in the water column, but they are also capable of feeding at the surface and on the substrate (McClure et al., 2006; Spence et al., 2007).

Compared to most cultured species, zebrafish have a shorter life cycle, reaching reproductive maturity after 3 months of age. It is an asynchronous fish, displaying an ovary which contains follicles at all stages of development (Clelland & Peng, 2009). In the wild, zebrafish follow a reproductive seasonal cycle, with the spawning period that occurs during high temperatures and food availability (before the monsoon in August) (Talling et al., 1998). However, under laboratory conditions they reproduce throughout the year, with spawning occurring typically every 4–7 days, exhibiting better spawning rates every 10 days (Clelland & Peng, 2009; Niimi & LaHam, 1974). Under controlled conditions, the maximum spawning activity occurs in the first 30 minutes after the light onset and continues for about an hour (Darrow & Harris, 2004). During spawning phase, zebrafish females can produce several hundred eggs in one clutch, ranging from 100 to 1000, with an average of 300 eggs (Lawrence, 2011; Ribas & Piferrer, 2014). Fertilization occurs outside, and adults have no parental care of the eggs, which are deposited over the substratum with aquatic vegetation and, preferably, in shallow waters (Sessa et al., 2008).

Within the same clutch, hatching occurs during the first 72 hours post-fertilization (hpf) (Kimmel et al., 1995). When incubated at constant temperature, at 28 °C, hatching rate of fertilized eggs is approximately 80% at 50 h post-fertilization (hpf), and finish at 76 hpf, during the light phase (independently of the photoperiod treatment) (Ribas & Piferrer, 2014). Metamorphosis occurs at the end of larval stage, which generally lasts up to 14 days. This phase involves changes in a variety of traits, including the absorption of the larval fins, the more ventrally dropping of the gut tube and the start of the development of scales with pigmentation patterns (Ledent, 2002). The juvenile stage is reached approximately after 28 dpf, when the pigment dorsal pattern is formed (Parichy & Turner, 2003). Juvenile fish reach puberty at approximately 45 dpf, growing exponentially over this period (W. Chen & Ge, 2013). Both wild and domesticated zebrafish typically achieve reproductive maturity at similar sizes, usually between 3 and 4 months of age, occasionally at 2 months, and the average lifespan is 3 years (Gerhard et al., 2002; Lawrence, et al., 2012).

Despite the high dietary adaptability, zebrafish possess a relatively simple morphology of the digestive tract. The zebrafish intestine comprises a single elongated tube that folds twice within the abdominal cavity (Menke et al., 2011). No distinct stomach, small intestine, or large intestine can be distinguished. However, differences can be observed in the morphology of the mucosa epithelial cells and in the abundance of goblet cells, indicating a functional differentiation (Menke et al., 2011). The intestinal epithelium is predominantly composed of columnar-shaped absorptive enterocytes, followed by goblet cells, and the several mucosal folds of intestine become progressively shorter towards the caudal side.

The zebrafish liver consists of three lobes located along the intestinal tract, and plays a crucial role in maintaining metabolic homeostasis, similarly to the mammalian one (Roberts, 2001). This encompasses the processing of nutrient and detoxification, along with the synthesis of serum proteins (Menke et al., 2011).

In contrast to the mammalian, the zebrafish liver lacks distinct organization of hepatocytes into cords or lobules and does not display typical portal triads; additionally, Kupffer cells are absent. Considering the biliary system,

the bile ducts fuse together forming the gallbladder, which contains greenish bile that is transported to the intestine through the common bile duct.

Zebrafish is a widely accepted animal model for a wide range of research areas, ranging from biomedicine to toxicology, including finfish aquaculture research (Ribas & Piferrer, 2014; P. E. Ulloa et al., 2014). It allows addressing all aspects of the life cycle including nutrition, welfare, and reproduction. Several studies in zebrafish focused on enhancing husbandry techniques, increasing survival rates, improving immune responses, optimizing nutrition, and promoting growth, aiming for relevant findings applicable to commercial fish species (Hedrera et al., 2013; Oyarbide et al., 2012; P. E. Ulloa et al., 2013).

A reliable fish model for aquaculture research must meet several essential criteria, such as: (i) exhibiting basic biological characteristics and physiological responses comparable to those of the most important farmed species; (ii) it should possess a short life cycle and being simple and cost-effective to breed; (iii) abundant resources should be available such as strains, genomic resources, lines, and mutants, to support research in different areas (Ribas & Piferrer, 2014).

It has been demonstrated that zebrafish, more than any other species, largely fulfils all these conditions, offering several advantages. It is considered to be representative of a larger group of organisms, being at the same time easier to handle and to study than the animals it is intended to represent (Ribas & Piferrer, 2014). In fact, it is an optimal model organism due to small size, ease of breeding and manipulation, short generation intervals, transparency of embryos, rapid early development, and high reproductive capacity (Aleström et al., 2006; Kimmel, 1989). These characteristics, for example, allow to perform all analyses with a high number of specimens per data point, standing in contrast to cultured fish species, which often have longer generation intervals (P. E. Ulloa et al., 2011, 2014). Moreover, since the reproductive system is well preserved across all fish species, zebrafish is a useful model for a comprehensive understanding mechanism involved in reproduction, helping to solve the breeding-related issues in cultured species (Weltzien et al., 2004).

Zebrafish has been also proposed as a model for studies on finfish nutrition and growth (P. E. Ulloa et al., 2011, 2014). In fact, despite numerous species-specific differences exist in various aspects of growth and nutrition, zebrafish exhibit an omnivorous diet, with nutritional pathways similar to those of farmed herbivores, such as carp and tilapia, as well as carnivores such as trout or salmon (P. E. Ulloa et al., 2011).

Compared with cultured fish, zebrafish possess a broad range of molecular tools and information available for genomic analysis, and a completely characterized genome. Indeed, the most powerful genomic resource of zebrafish is the accessibility of its complete genome sequence (Kocher & Kole, 2008; https://www.ncbi.nlm.nih.gov/grc/zebrafish), which exhibits synteny with the human one (Barbazuk et al., 2000), as well as with the salmonid genomes, aiding the identification of disease-associated genes, simplifying tests and genetic screening (Quinn et al., 2008; Rexroad et al., 2008; P. E. Ulloa et al., 2011). Finally, a rapid development of genetic and genomics resources has been made recently for economically important cultured fish species, such as channel catfish (P. Xu et al., 2007), rainbow trout (Rexroad et al., 2003), atlantic salmon (Davidson et al., 2010), atlantic cod (*Gadus morhua*) (Johansen et al., 2009) and gilthead sea bream (*Sparus aurata*) (Sarropoulou et al., 2007).

European sea bass

Class: Actinopterygii
Order: Perciformes
Family: Moronidae
Genus: *Dicentrarchus*
Species: *Dicentrarchus labrax*



The European sea bass (*Dicentrarchus labrax*) is a marine teleost belonging to the Moronidae family, and it is a key species for both marine biology and commercial aquaculture (Vandeputte et al., 2019). It is distributed throughout the north-eastern Atlantic Ocean, up to the Mediterranean and the Black Sea. European sea bass usually live in shallow waters (<100 m), inhabiting diverse environments such as coastal waters, estuaries, and even brackish inland waters. Especially at the juvenile stage, it is often found foraging in estuaries and lagoons from spring to fall. In fact, it is both a euryhaline and eurythermal species, which can tolerate a salinity of 0-40 ppt and a water temperature of 2-32 °C (Vandeputte et al., 2019).

European sea bass males typically reach maturity between the ages of two to three years, while females typically mature between three to four years of age (Pérez-Ruzafa & Marcos, 2014). Females have an average spawning rate of 200,000 eggs/kg, with external fertilization (Vandeputte et al., 2019). Hatching typically occurs within three to five days post-fertilization, and the larvae, 4 mm upon hatching, progress to the post-larval stage (>22 mm) within two to three months.

The European sea bass is an opportunistic carnivores, feeding on plankton at the larval stage, while on fish, crustaceans, and cephalopods at the juvenile and adult stages (Vandeputte et al., 2019). The high dietary flexibility of this fish contributes to its adaptability and success in various habitats.

The anatomy of the digestive tract is similar to those of the majority of teleost, in which can be distinguished an anterior (proximal) intestinal segment, mid, and posterior (or distal) intestine (Ferreira et al., 2023; Torrecillas et al., 2019). The proximal intestine ranges from the pyloric caeca to the ileorectal valve, while the mid and distal segments are located between the ileorectal valve and the rectum. The anterior region displays primarily absorptive functions, with largest absorptive area, longest mucosal folds, and a high expression of absorption- and digestion-related genes (Torrecillas et al., 2019). In contrast, both distal intestine and rectum display immunity functions.

The liver presents similarity with those of mammalian, despite the absence of proper lobulation, and the hepatocytes are arranged as cords, which extend between central and portal zones (Vicentini et al., 2005).

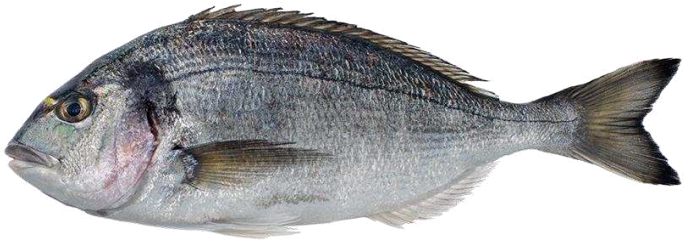
European sea bass is also a key species in aquaculture, particularly in Mediterranean area, which accounts for 94% of the production (mostly in Turkey, Greece, Egypt and Spain). It was the first non-salmonid marine species commercially cultured in Europe (https://www.fao.org/fishery/en/culturedspecies/dicentrarchus_labrax/en).

Aquaculture production of European sea bass involves two distinct phases. Initially, during the hatchery-pre-growing phase, fish weigh between 1 to 20 grams and are produced over a three- to eight-month period (Vandeputte et al., 2019). Subsequently, during on-growing phase, fish are raised up to a weight of 250 to 450 grams within 12 to 20 months. Hatcheries are typically inland and utilize temperature-controlled systems to provide optimal conditions for the early growth stages of the fish (Kousoulaki et al., 2015; Vandeputte et al., 2019). In contrast, the majority of the on-growing phase takes place in sea cages, located in natural waters. This approach allows for efficient utilization of marine resources and provides an environment conducive to the growth and development of fish. The main market product is the 250 – 400 g pan-sized fish, but there is growing interest in the production of larger fish (800 g to 1 kg) to sell whole or as processed fillets (EUMOFA 2018).

As in all farmed fish species, growth is a crucial trait, particularly considering that sea bass takes 18 to 24 months to reach commercial size. The sex determination in this species follows a polygenic system, where both genetics and environmental factors play significant roles (Vandeputte & Piferrer, 2018). During larval phase, rearing temperature has a substantial influence on sex differentiation, and since hatcheries often employ higher temperatures to accelerate growth, farmed populations tend to consist predominantly of males (Saillant et al., 2001). However, this poses challenges for the production, as male sea bass typically grow 20%–30% less than females and exhibit early maturation, both of which are unfavorable traits for aquaculture (Saillant et al., 2001; Vandeputte et al., 2019).

Gilthead sea bream

Class: Actinopterygii
Order: Acanthuriformes
Family: Sparidae
Genus: *Sparus*
Species: *Sparus aurata*



The gilthead sea bream (*Sparus aurata*) is a perciform fish which belongs to the Sparidae family. It is commonly distributed in the Mediterranean Sea, along the Eastern Atlantic coasts from Great Britain to Senegal, and rarely in the Black Sea (Arabaci et al., 2010). It is characterized by an oval and laterally compressed body, with a prominent silver coloration with a notable golden band between the eyes, giving rise to its common name.

Due to its euryhaline and eurythermal habits, the species is adapted to a variety of environments, despite being highly sensitive to low temperatures, with a lower lethal threshold of 4 °C (FAO, 2005). Its habitats range from marine to brackish waters, comprising rocky coastal reefs, sandy bottoms and seagrass beds, as well as lagoons and estuaries, especially during early life stages. Juveniles often inhabit shallow and sheltered waters (30 m maximum), moving to deeper areas as they mature. Adults reach depths of up to 50 m, although specimens up to a depth of 150 m have been reported.

Males reach sexual maturity at the age of 2 years, measuring 20-30 cm in length, while females reach maturity at the ages from 2 to 3 years, measuring between 33 and 40 cm (FAO, 2005; Zohar et al., 1978). This species exhibits protandrous hermaphroditism, typically starting life as males and later transitioning into females.

Indeed, between 4 and 8 months of age, an early development of the bisexual gonad can be observed (Zohar et al., 1978). At the age of 4 months, the gonad is undifferentiated, while between 13 and 16 months the sex reversal process begins. During this period, the testicular part of the gonad is spent and remains latent, while the ovarian part increase in growth and the oogonia start to undergo oogenesis.

Females present asynchronous ovarian and are batch spawners, laying 20 to 80 thousand eggs per day, up to 3-4 months (FAO, 2005). In the wild, spawning occurs in the late autumn and winter, and eggs are released into the water column where external fertilization occurs. The eggs are spherical and pelagic, measuring slightly less than 1 mm in diameter, and contain a single large oil droplet (Arabaci et al., 2010). The larvae are planktonic, drifting with the currents until they settle in suitable habitats. Sea breams born in the open sea, and as juveniles, they typically migrate towards protected coastal waters during early spring (FAO, 2005). These areas provide abundant trophic resources and milder temperatures. During October-December, adult fish return to the open sea, where breeding occurs.

The gilthead seabream is a mainly carnivorous fish, feeding on crustaceans such as mussels, oysters, crabs and shrimp, although it can be accessorily herbivorous (FAO, 2005). It possesses strong jaws, which present specialized canine-like teeth anteriorly, followed by blunter teeth posteriorly, that allow it to crush hard shells,

making it a versatile feeder. The esophagus enters the Y-shaped stomach, the bottom of which is very extensible, without any external differentiation (Cataldi et al., 1987). At the beginning of the intestine, four short pyloric caeca are present, at the base of which the ductus hepaticus and the *ductus pancreaticus* discharge. The pyloric appendices present a simple columnar epithelium, with microvilli that are more abundant and longer compared to the intestine. The muscularis and submucosa are very thin in juveniles but develop in the adult. The digestive canal forms two bends.

The intestine mucosa, typically folded, is composed of columnar, mono-stratified enterocytes with their lumen surfaces entirely covered by microvilli (Cataldi et al., 1987). Numerous mucus-secreting cells are present in the epithelium, which increase in number in caudal region of the intestine. In this latter portion, the last valve presents enterocytes displaying a different aspect, containing many vacuoles above the nucleus, that are rich with large and refracting eosinophilic granules.

The liver consists of two lobes, which are not completely separated, and the larger left lobe protrudes posteriorly. Liver is formed by a network of epithelial cells, through which runs vessels and sinusoids. The ductus hepaticus enters the first part of the intestine, after running parallel to a short *ductus pancreaticus*.

Gilthead sea bream is of great economic importance in Mediterranean aquaculture. This fish species can be farmed with extensive and semi-intensive methods, within coastal ponds and lagoons, as well as with intensive methods within land-based installations and sea cages (FAO, 2005).

Considering the first two systems, the sea bream takes up to 20 months to reach its first commercial size (350 g). The total production of this kind of polyculture can vary widely (up to 30kg/ha/yr and up to 2400 kg/ha/yr, for extensive and semi-intensive, respectively) according to lagoon productivity or the size of the juveniles stocked, as well as the amount of feed provided (FAO, 2005). The fish density generally does not exceed high values (0.0025 kg/m³ and 1 kg/m³ for extensive and semi-intensive, respectively).

In intensive tank rearing methods, gilthead seabreams are raised at very high densities, typically ranging from 15 to 45 kg/m³. To ensure fish survival, significant oxygen injections are required. Under optimal conditions (rearing temperature between 18-26°C), pre-fattened small gilthead seabreams (5 g) can reach commercial size (350-400 g) in approximately one year. Considering sea cages, densities (10-15 kg/m³) are lower than in tanks, and no energy costs for pumping, aeration, or post-rearing water treatment are necessary (FAO, 2005). However, temperature control is not possible in cage farming, resulting in a longer rearing period to reach market size (16 months). The white and firm flesh are highly appreciated for their taste and nutritional qualities, making the gilthead sea bream a popular choice in Mediterranean cuisine. Because of its economic and ecological importance, sustainable farming practices are increasingly important to maintain healthy populations and ecosystems.

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Chapter 2

The Application of Synthetic Flavors in Zebrafish (*Danio rerio*) Rearing with Emphasis on Attractive Ones: Effects on Fish Development, Welfare, and Appetite

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Abstract

The aim of the present study was to test synthetic flavors as potential feed attractants in zebrafish (*Danio rerio*) during early development. Six experimental groups were set up in triplicate: (i) a CTRL group fed a zebrafish commercial diet; (ii) a PG group fed a control diet added with Propylene Glycol (PG); (iii) $A1^+$ and $A2^+$ groups fed a control diet added with 1% of the two attractive flavors ($A1^+$ cheese odor made by mixing Propylene Glycol (PG) with the aromatic chemicals trimethyamine, 2-acetylpyrazine, 2-acetylpyridine, and dimethyl sulfide; and $A2^+$ caramel odor, made of PG mixed with the aromatic chemicals vanillin, maltol, cyclotene, acetoin, butyric acid, and capric acid with traces of both gamma-octalactone and gamma-esalactone) or the repulsive flavor (A^- coconut odor, made by mixing PG with the aromatic chemicals gamma-eptalactone, gamma-nonalactone, delta-esalactone, and vanillin with trace of both delta-octalactone and maltol), respectively; (iv) an ROT group fed the two attractive diets, each administered singularly in a weekly rotation scheme. All the tested synthetic flavors did not affect the overall health of larval and juvenile fish and promoted growth. Due to the longer exposure time, results obtained from the juvenile stage provided a clearer picture of the fish responses: zebrafish fed both attractive diets showed higher appetite stimulus, feed ingestion, and growth, while the brain dopaminergic activity suggested the $A2^+$ diet as the most valuable solution for its long-

lasting effect over the whole experiment (60-day feeding trial, from larvae to adults). The present study provided important results about the possible use of attractive synthetic flavors for aquafeed production, opening new sustainable and more economically valuable opportunities for the aquaculture sector.

Introduction

In the past few decades, aquaculture has significantly increased in terms of food production. For instance, from 2010 to 2020, this sector globally grew at an average annual rate of 4.6%, reaching a record of 122.6 million tons in 2020, including 87.5 million tons of aquatic animals worth USD 264.8 billion [1]. This is largely due to the food demand of an expanding world population, which is estimated to reach 9 billion people by 2050. Human fish consumption has more than doubled in the past 50 years, reaching 20.2 kg per capita in 2020 [1]. However, to ensure further development of the aquaculture sector, proper resource management is required to promote sustainability and circularity [2], with emphasis on the aquafeed production sector, which still relies on the use of conventional marine- and plant-derived raw materials [3]. Fish meal (FM) and fish oil (FO) represent the ideal ingredients for aquafeeds, ensuring proper fish growth and welfare [4], but due to their increasing costs and ecological drawbacks, they have been gradually replaced, over the last few decades, by plant-derived ones [5]. However, the use of plant protein sources like soybean or corn meals for fish feed formulations, besides leading to food *vs*. feed competition, presents several negative side effects on fish growth and welfare, especially for carnivorous species [6,7]. The presence of nondigestible carbohydrates and antinutritional factors can adversely affect nutrient digestibility/bioavailability, and consequently, fish health status [8], in addition to often being poorly palatable [3]. Part of these problems has been addressed by (i) improving the processing methods of plant-derived ingredients (protein hydrolysates; inclusion of feed supplements such as butyrate) [9,10]; (ii) replacing plant-derived ingredients with promising and more sustainable alternatives of animal origin like poultry by-product, insect, and crayfish meals or proteins derived from microbial biomasses, including fungi, microalgae, and cyanobacteria [11–14]. However, the production of more sustainable diets often results in lower diet palatability, affecting fish growth, water quality, and farm economics [15]. In fact, it is estimated that a considerable portion of feed administered in fish farms is not ingested by fish, ending in wastewater outflows and contributing to eutrophication of aquatic ecosystem and producing economic losses [16–18]. Nowadays, a class of feed additives, known as feed attractants, are regularly included in aquafeeds to improve nutrient-sensing of fish [19]. Chemical stimuli originated from highly palatable feed can influence both feed-seeking and ingestion, by activating the gustatory and olfactory senses of fish [20]. In vertebrates, feeding is a complex mechanism controlled by a fine brain–gut crosstalk mediated by neurohormonal signals [21]. The appetite stimulus is induced by different orexigenic signals, among which ghrelin and neuropeptide Y (NPY) have been widely demonstrated to play an essential role in stimulating feeding in teleost [22]. Ghrelin is directly produced by the entero-endocrine cells of the gut tract

and possesses a strong orexigenic role promoting, at a central level, the expression of *npy* [23,24]. Conversely, a plethora of anorexigenic signals both produced by central and peripheral tissues play a major role in suppressing feeding [22]. In this context, leptin decreases the feeding intake by inhibiting the orexigenic signals and stimulating the anorexigenic pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) system at central level [25] and induces the mobilization of energy reserves by stimulating lipolysis [23,26]. Fish feeding behavior and appetite sensation are also closely related to the brain reward system, in which dopamine is the main neurotransmitter involved in the hedonic appetite regulation, that increases motivation after the ingestion of palatable feed [26,27]. Thus, improving the organoleptic properties of fish diets through the use of specific feed attractants represents a critical aspect to elicit an optimal feeding response by fish in terms of time and feed intake [10]. Nowadays, feed attractants for aquafeeds are mainly represented by natural-derived ingredients [28]. Meals obtained from shrimp, anchovy, and squid are well-recognized feed attractants, regularly included in fish diets to improve palatability [29,30]. On this regard, Kader et al. [31] tested the effects of fish soluble (*Katsuwonus pelamis* by-product), krill meal, squid meal, and crystalline amino acids as feed attractants in a plant-based diets on red seabream, *Pagrus major*, concluding that all of these ingredients acted as feed attractants. Additionally, Nagel et al. [30] investigated the potential of blue mussel meal as feed attractant in a plant-based diets for turbot (*Psetta maxima*), observing similar results. All of these solutions play a positive role as feed attractants, but their production still depends on marine resources, posing further unsustainability issues for the aquafeed production sector [32]. In addition, due to their natural origin, these ingredients present fluctuations in availability and their attractive effect is highly variable depending on the raw material composition, freshness, and processing methods [33]. The current alternative solutions that have been tested are represented by a limited number of molecules, such as a mixture of free amino acids (e.g., glycine, L-alanine), nucleosides and nucleotides, or substances such as betaine and taurine, which can be used as feed attractants [34–36]. Marine extracts are rich in all these compounds, although amino acids appear to be the main class of molecules that induce feed-seeking behavior in fish [37]. Amino acids have been widely studied for their attractive properties, although the limitations and disadvantages of their use as feed attractants are well known [38]. It is noteworthy that free amino acids exhibit different attractive effects depending both on the fish species and the life cycle stage considered, thus leading to controversial results. Furthermore, the attractive effect depends also on the amino acid used as well as on its concentration and interaction with other molecules present in the diets. Finally, there is a lack of data in the scientific literature on the identification of optimal dosage for different fish species, and anyway, the application of amino acids as feed attractants is less effective than the above-mentioned marine-derived ingredients [36,38]. Therefore, a novel, standardized, and sustainable alternative to natural feed attractants may be represented by synthetic flavors, obtained through standardized processes. The available literature provides only few specific studies on this topic [28], which instead can represent a great opportunity for the aquaculture sector to satisfy the organoleptic requirements of fish [39]. Indeed, it is well known that in the food industry, a limited number of molecules allow us to replicate specific flavors [40]. A synthetic flavor is composed by a mixture of aromatic chemicals such as esters, terpenes, aldehydes, phenols, lactones, fatty acids, and sulfur

compounds [41], recognized as safe for use by the American Flavor and Extracts Manufacturers Association (FEMA). These substances are combined in a liquid blend with a functional solvent (e.g., water, triacetin, propylene glycol) to impart or improve a particular flavor (e.g., banana, cherry, chocolate) to different products [39,41]. In this way, thousands of different food-related flavors can be faithfully reproduced through sustainable and cost-effective processes [42]. In this context, the aim of the present study was to identify different synthetic flavors that are widely used for human food consumption, and to test them during zebrafish (*Danio rerio*) culture to assess their potential role as feed attractants. Zebrafish represents a well-validated model for aquaculture nutritional studies and allows us to investigate possible dietary effects on all its life stages in a relatively short time [43–46]. A set of positive and negative commercial synthetic flavors were initially identified through a behavioral preference test and secondly added to a specific zebrafish diet which was administered to fish from the larval to the juvenile stage. At the end of both larval and juvenile phases, biological samples were collected and analyzed through a multidisciplinary approach to obtain a comprehensive overview of the fish physiological responses to the synthetic flavors here tested.

Methods

Ethics

All the experimental procedures involving animals conducted in the present study were performed in accordance with the Italian legislation on experimental animals and were approved (n° 2-1/12/22) by the Ethics Committee of the Marche Polytechnic University (Ancona, Italy) and the Italian Ministry of Health (Aut N° 453/2023-PR). An anesthetic (MS222 1 g/L; Merck KGaA, Darmstad, Germany), was used to minimize the suffering of the animals.

Synthetic Flavors

Forty commercial flavors were produced and blindly provided by To Be Pharma S.r.l. (Via Vibrata 127, 64016, S. Egidio alla Vibrata, Teramo, Italy). All the 40 synthetic flavors tested were composed by the same basic solvent (1,2-propanediol, Propylene Glycol) and a mixture of different flavoring chemicals, produced through a standardized process and widely used for human consumption [39,41]; Flavor and Extracts Manufacturers Association—FEMA. The use of Propylene Glycol has also been classified as safe for use by Food and Drug Administration (FDA; Agency for Toxic Substances and Disease Registry, 2007). As regards the selected flavors for the present study, the F25 one was composed of Propylene Glycol (PG) as solvent mixed with the following aromatic chemicals: trimethylamine, 2-acetylpyrazine, 2-acetylpyridine, and dimethyl sulfide. Differently, the F35 flavor was composed by PG as solvent, mixed with the aromatic chemicals vanillin, maltol, cyclotene, acetoin, butyric acid, and capric acid with traces of both gamma-octalactone and gamma-esalactone. Finally, the F32 flavor was composed by PG, as solvent mixed with the aromatic chemicals gamma-eptalactone, gamma-nonalactone, delta-esalactone, and vanillin with traces of both delta-octalactone and maltol. The final odors of F25, F35, and F32 flavors were cheese, caramel, and coconut, respectively. Due to intellectual property protection, the composition of each single tested flavor in the present study is reported as

relative abundance of each single chemical (first one more abundant, last one less abundant). All products tested were produced with nontoxic food flavorings developed for human consumption in agreement with the current sector-specific legislation (Regulation (EC) No 1334/2008 and 1333/2008) and are safe for humans and animals, as reported from the technical data sheet and the Material Safety Data Sheet (MSDS).

Preliminary Behavioral Preference Test

All 40 synthetic flavors were tested through a behavioral preference assay conducted on zebrafish larvae following a previously published protocol developed for this species [47]. This preliminary study was performed to identify the flavors determining a significant behavioral response (i.e., attraction or avoidance) to be used in the following analyses of the study. Each flavor was presented to 20 individual zebrafish larvae (N = 800 larvae in total; N = 20 replicates per flavor). The larvae were obtained through a standard breeding protocol from groups of adult zebrafish of the AB strain. In detail, the adults were kept in spawning tanks with a bottom grid that permitted to collect the eggs upon fertilization and avoid cannibalism. Eggs were collected from multiple spawns of different adult zebrafish. After collection, the eggs were maintained in small groups in Petri dishes until hatching, and then moved into a Tecniplast system (Varese, Italy) with continuous water circulation and filtration until the behavioral test. For the testing, subjects from the pool of zebrafish larvae (21 days postfertilization; dpf) were randomly selected and were individually moved into 8 × 24 cm white plastic tanks, filled with 0.5 L of clean water. Multiple experimental tanks were used to simultaneously test multiple subjects. After 30 min of acclimatization, in each tank, a cellulose sponge (1 cm^3) soaked with one of the 40 flavors was introduced. The specific flavor was randomly assigned to each subject. The sponge with the stimulus was placed in correspondence of one of the shorter sides of the testing tank, kept in place close to the bottom by a small pin. The flavors were diluted 1:100 with distilled water. Additionally, a second sponge soaked with distilled water only was used as control and located on the opposite side of the flavor-soaked sponge. The behavior of the subject was video-recorded thanks to cameras placed above the tanks (Sony LEGRIA HFR38) using tripods. The test duration was 15 min, during which each subject was left free to swim. After each test, the tanks were completely washed with distilled water and refilled with clean water before testing another subject. The video recordings were successively analyzed using BORIS software (ver. 7.12.2) [47]. This allowed us to calculate the time spent by each larva close to the stimulus (flavored sponge) or close to the control sponge, for each minute of the test. As detection threshold, an area of 1/3 of the experimental tank for each stimulus (8 × 8 cm) was used. These data were managed to compute an index of percentage preference for the flavor controlled for the control sponge as follows: (time close to the flavor/(time close to the flavor + time close to the control sponge)) × 100. This index allowed us to exclude time spent by the subject far from both stimuli (i.e., in the center of the experimental tank). The index ranged from 0 to 100%, assuming different values according to the preference of the subjects: it showed values higher than 50% when a subject spent most of the time close to the flavor; conversely, the index had values smaller than 50% when a subject spent most of the time close to the control sponge with no flavor. In case of no preference, the value of the

index was close to 50%. The index was calculated in two-time blocks of 0–5 and 0–15 min, respectively, because this preference usually varies across the testing time [47].

Production of Experimental Diets

Starting from a commercial diet for zebrafish (Zebrafeed; Sparos LDA, Olhão, Portugal), used as control (CTRL diet) (for composition, please see [48]), four experimental diets were prepared as follows: (i) PG diet obtained by adding 1% (w/w) of Propylene Glycol (the basic solvent of the flavors; PG) to the control diet; (ii) $A1^+$ diet obtained by adding 1% (w/w) of F25 as attractive/positive flavor to the control diet; (iii) $A2^+$ diet obtained by adding 1% (w/w) of F35 as attractive/positive flavor to the control diet; (iv) A^- diet obtained by adding 1% (w/w) of F32 as repulsive/negative flavor to the control diet. Flavors and PG were added daily to the right amount of the control diet at 1% (w/w) using a micropipette and vigorously mixed to guarantee a homogenous distribution in the feed.

Experimental Design

Zebrafish AB strain adults fed on a commercial diet for zebrafish (Zebrafeed; Sparos LDA, Olhão, Portugal) were laboratory-spawned and the obtained embryos were collected and maintained according to Cattaneo et al. [49]. After 48 hrs of embryo development, a total number of 9000 live and developing embryos were selected under a stereomicroscope, collected, and then randomly divided into six experimental groups (3 tanks per experimental group): (i) CTRL group, zebrafish fed the control diet); (ii) PG group, zebrafish fed control diet added with 1% of Propylene Glycol (PG diet); (iii) $A1^+$ group, zebrafish fed control diet added with 1% of positive/attractive flavor (diet $A1^+$); (iv) $A2^+$ group, zebrafish fed control diet added with 1% of positive/attractive flavor (diet $A2^+$); (v) A^- group, zebrafish fed control diet added with 1% of the negative/repulsive flavor (A^- diet); (vi) ROT group, zebrafish fed the two attractive diets ($A1^+$ and $A2^+$), each administered singularly in a weekly rotation scheme. This last group was included in the experimental design since it has been demonstrated that teleost, in response to a repeated stimulation, may develop an olfactory receptors adaptation [50]. Zebrafish larvae were initially reared in 20 L tanks (500 larvae per tank, 3 tanks per experimental group) with the same water conditions of the broodstock's tanks and provided by a dripping system to ensure a proper water change and black panels placed on the sides of each tank to reduce light reflection [51]. After 21 days postfertilization (dpf), zebrafish from each tank were gently moved to other tanks (100 L, 3 tanks per experimental group) provided with mechanical and biological filtration (Panaque, Capranica, Italy). Starting from day 5 dpf to the end of the trial (60 dpf), zebrafish were fed the experimental diets, tanks were cleaned and dead specimens collected and counted, according to Rashidian et al. [52] and Zarantoniello et al. [48]. The required number of fish per tank was sampled, after a lethal dose of MS222 (1 g/L, Merck KGaA, Darmstad, Germany), at 21 dpf to evaluate dietary effect of flavors administration during the larval development. The remaining fish were sampled at 60 dpf, after being euthanized with a lethal dose of MS222 (1 g/L, Merck KGaA), to evaluate the long-term dietary effect of flavor administration on juvenile stage.

Biometry

Ten just-hatched larvae per tank (30 per dietary group) were randomly collected at 3 dpf to measure the initial body weight (IBW). Then, 20 zebrafish larvae and 20 zebrafish juveniles (60 larvae and 60 juveniles per experimental group) were randomly collected from each tank at 21 and 60 dpf, respectively, to measure the final body weight (FBW). The specific growth rate (SGR) was calculated as follows:

$$SGR = [(\ln FBW \times \ln IBW)/t] \times 100 \quad (1)$$

in which t represents the number of feeding days (17 and 57 for larvae and juveniles, respectively). The survival rate was calculated at 21 and 60 dpf for both the developmental stages, by deleting the dead specimens to the initial number of fish.

Feed Intake

The experiment was conducted only in the juvenile stage since, for the larval stage, the sizes of both pellets and fish were too small to have an accurate result. Fifteen zebrafish juveniles per tank (45 per experimental group) were acclimatized for one week prior to the feed intake experiment and were fed the experimental diets, providing a pre-weighed quantity of feed corresponding to 3% of their body weight. During the feed intake experiment, eventually, uneaten feed was recovered 15 min post-administration by siphoning. The recovered uneaten feed was dried in an oven overnight at 40 °C for quantification. The duration of the test was selected according to the maximum duration of the behavioral preference test for flavor selection (15 min).

Histological Analysis

Five whole larvae per tank (15 per experimental group) were collected at 21 dpf, and liver and whole intestine samples from 5 juveniles per tank (15 per experimental group) were collected at 60 dpf according to Cattaneo et al. [49]. Samples were fixed by immersion in Bouin's solution (Merck KGaA) for 24 h at 4 C and then washed three times with 70% ethanol for 15 min and maintained in a new 70% ethanol solution at 4 C. Using graded ethanol solutions (80, 95, and 100%) samples were dehydrated, then washed with xylene (Bio-Optica, Milano, Italy), and finally embedded in paraffin (Bio-Optica). Using a microtome (Leica RM RTS, Nussloch, Germany), sections of 5 m were cut from the solidified paraffin blocks. Sections were stained with Mayer hematoxylin and eosin Y (Merck KGaA) to estimate possible alterations in tissue architecture and presence of inflammations in both the intestinal tract and the liver. The evaluation of histological indexes in the intestine was performed on three transversal sections per fish (15 whole larvae and 15 intestines from juveniles per experimental group) collected at 50 µm intervals according to [49,53]. Specifically, for the morphometric evaluation of mucosal fold height, all the undamaged and non-oblique folds were measured using ZEN 2.3 software (Zeiss, Oberkochen, Germany). Regarding the analysis of histopathological indexes, scores were assigned as follows: (i) inflammatory influx: + = scarce lymphocyte infiltration, ++ = moderate lymphocyte infiltration, +++ = diffused lymphocyte infiltration; (ii) mucosal folds fusion: + = 0–3 observations per section, ++ = 3–10 observations per section, +++ > 10 observations per section.

Molecular Analyses

Briefly, total RNA extraction and cDNA synthesis from 3 larvae per tank (in triplicate, 9 larvae per group) collected at 21 dpf, and from brain, liver, and whole intestine samples from 3 juveniles per tank (in triplicate, 9 brains, 9 livers, and 9 intestines per group), collected at 60 dpf, were performed according to Cattaneo et al. [49] and Olivotto et al. [54]. Real-time quantitative PCR (qPCR) reactions were performed in an iQ5 iCycler thermal cycler (Bio-Rad, Hercules, CA, USA) on a 96-well plate according to Randazzo et al. [55], including a nonreverse transcription control for each reaction to exclude possible contamination by genomic DNA. For each sample, reactions were set according to Cattaneo et al. [49]. The thermal profile was 3 min at 95 °C, followed by 45 cycles of 20 s at 95 °C, 20 s at the melting temperature for each primer, as reported in Table 1, and 20 s at 72 °C. Annealing temperatures for each primer were optimized through temperature gradient assays, and primer specificities were assessed with the absence of primer–dimer formation and dissociation curves. Additionally, for each primer, efficiencies were evaluated with a mix of cDNA from CTRL group at different concentrations (1:1, 1:10, 1:100, 1:1000); for all the primers tested, efficiency was around 90%, with an R^2 that ranged from 0.995 to 0.998. At the end of each cycle, the fluorescence was monitored, and the melting curve analyses showed one single peak for every product. Absence of contamination and no peaks were found in the two no-template controls (NTCs) for each reaction. Relative mRNA abundance was used to quantify the gene expression using, as housekeeping genes, the ribosomal protein L13 (*rpl13*) and actin-related protein 2/3 complex subunit 1a (*arpc1a*) to standardize the results. The relative quantification of genes involved in fish growth (insulin-like growth factor, *igf1*; myostatin, *mstnb*), appetite regulation (ghrelin, *ghrl*; neuropeptide Y, *npy*; and leptin, *lepa*), brain reward system (dopamine receptor D1b, *drd1b*; dopamine receptor D2a, *drd2a*; dopamine receptor D3, *drd3*), stress response (glucocorticoid receptor, *nr3c1*), and immune response (interleukin 1, *il1b*; interleukin 10, *il10*; lipopolysaccharide-induced TNF factor, *litaf*) was performed on samples from pool of whole larvae or from target organs considering juveniles. Particularly, (i) *igf1*, *mstnb*, *nr3c1*, and *lepa* were tested in liver samples; (ii) *ghrl*, *il1b*, *il10*, and *litaf* were tested in intestine samples; (iii) *npy*, *drd1b*, *drd2a*, and *drd3* were tested in brain samples. Amplification products were sequenced, and homology was verified. Gene transcript expression alterations among experimental groups are reported as relative mRNA abundance (arbitrary units). The qPCR data were processed using iQ5 optical system software version 2.0 (Bio-Rad), including GeneEx Macro iQ5 Conversion and genex Macro iQ5 files.

Table 1. Sequences, annealing temperature (AT), and accession number of primers used in the present study.

Gene	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	AT (°C)	ID
igf1	GGCAAATCTCCACGATCTCTAC	CGGTTTCTCTTGTCTCTCTCAG	53	ZDB-GENE-010607-2
mstnb	GGACTGGACTGCGATGAG	GATGGGTGTGGGGATACTTC	58	ZDB-GENE-990415-165
ghrl	CAGCATGTTTCTGCTCCTGTG	TCTTCTGCCCACTCTTGGTG	58	ZDB-GENE-070622-2
npy	GTCTGCTTGGGGACTCTCAC	CGGGACTCTGTTTCACCAAT	60	ZDB-GENE-980526-438
lepa	CTCCAGTGACGAAGGCAACTT	GGGAAGGAGCCGGAAATGT	60	ZDB-GENE-081001-1
drd1b	CTGCGACTCCAGCCTTAATC	AGATGCGGGTGTAAGTGACC	58	ZDB-GENE-070524-2
drd2a	TGGTACTCCGGAAAAGACG	ATCGGGATGGGTGCATTTC	58	ZDB-GENE-021119-2
drd3	ATCAGTATCGACAGGTATACAGC	CCAAACAGTAGAGGGCAGG	60	ZDB-GENE-021119-1
nr3c1	AGACCTTGGTCCCCTTCACT	CGCCTTTAATCATGGGAGAA	58	ZDB-GENE-050522-503
il1b	GCTGGGGATGTGGACTTC	GTGGATTGGGGTTTGATGTG	54	ZDB-GENE-040702-2
il10	ATTTGTGGAGGGCTTTCCTT	AGAGCTGTTGGCAGAATGGT	56	ZDB-GENE-051111-1
litaf	TTGTGGTGGGGTTTGATG	TTGGGGCATTTTATTTTGTAAG	53	ZDB-GENE-040704-23
rpl13	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG	59	ZDB-GENE-031007-1
arpc1a	CTGAACATCTCGCCCTTCTC	TAGCCGATCTGCAGACACAC	60	ZDB-GENE-040116-1

Total Lipid Analysis

Three juveniles per tank (in triplicate, 9 per experimental group) were collected at 60 dpf; then, they were manually cut, pooled together, and divided into two parts which were subdued to the lipid extraction using chloroform:methanol (2:1) as solvents following the method proposed by Folch et al. [56]. Once extracted, the amount of total lipids was gravimetrically quantified, and the results were expressed as g/100 g of whole fish. Total lipids were also extracted from the zebrafish larvae (15 larvae each group) following the same procedure described above.

Statistical Analyses

Data from each analysis were checked for normality (Shapiro–Wilk test) and homoscedasticity (Levene's test). The behavioral preference data were analyzed with a one-way analysis of variance (ANOVA) on the entire data set followed by one-sample t-tests (against random choice: 50%) for each individual flavor. All the data of the following experiments were then analyzed through ANOVA followed by Tukey's multiple comparison post hoc test, performed using the software package Prism 8 (GraphPad software version 8.0.2, San Diego, CA, USA). Significance was set at $p < 0.05$.

Results

Behavioral Preference Test

Behavioral preference test data were initially analyzed by comparing the index across all the flavor tested. This analysis revealed that the percentage of time spent by fish close to the stimulus varied significantly according to the flavor (ANOVA, $F_{39,724} = 2.42$, $p \leq 0.0001$, $\eta^2_g = 0.12$). This indicated that the subjects responded differently to the different flavors administered. To further investigate this differential response, we analyzed separately the preference for each flavor. Since in case of no preference, the index was expected to assume a value close to 50% (see Section 2/Methods), we compared the index of each flavor against this value using one-sample t-tests. The comparison was performed both considering the overall experiment (0–15 min) and the 0–5 min time block separately, given the evidence of behavioral changes across the testing time. Two of the flavors showed an attractive effect on zebrafish and were used as attractive flavors in the following stages of the study. Zebrafish exposed to flavor F25 spent significantly more time close to the flavor than to the control sponge, especially at the beginning of the test (index of preference for the flavor in the initial 5 min: 59.21 ± 17.85%; one-sample t test: $t_{18} = 2.250$; $p = 0.0372$). Moreover, flavor F35 determined a long-lasting attraction during the whole experimental time (60.80 ± 11.30%; $t_{18} = 4.165$; $p = 0.0006$). Conversely, one of the flavors determined a repulsive effect: subjects exposed to flavor F32 spent significantly less time close to the sponge with the flavor than close to the control one (42.50 ± 9.87%; $t_{18} = -3.314$; $p = 0.0039$). F32 was thus identified as repulsive flavor to be used in the following part of the study.

Biometry

As regard survival rate, no significant differences were evident among the experimental groups at both the larval (87 ± 3, 85 ± 6, 85 ± 4, 86 ± 6, 85 ± 6, and 85 ± 5% for CTRL, PG, $A1^+$, $A2^+$, ROT, and A^-, respectively) and juvenile (84 ± 4, 83 ± 5, 84 ± 5, 82 ± 4, 83 ± 6, and 83 ± 5% for CTRL, PG, $A1^+$, $A2^+$, ROT, and A^-, respectively) stages. Figure 1 shows the specific growth rate of both larvae and juveniles fed on the different diets. Considering larvae (Figure 1a), no significant differences were detected among all the groups fed the flavor-added diets (11.63 ± 0.85, 11.47 ± 1.95, 8.95 ± 1.94, and 8.26 ± 1.31% for the $A1^+$, $A2^+$, ROT, and A^- groups, respectively) and the CTRL and PG (9.15 ± 2.82 and 8.63 ± 1.90%) groups. However, larvae fed $A1^+$ and $A2^+$ diets were characterized by a significantly ($p < 0.05$) higher SGR value compared to those fed the A^- one. As regards juveniles (Figure 1b), no significant differences were detected between the CTRL and PG groups (9.08 ± 0.16 and 8.95 ± 0.17%). Fish fed $A1^+$, $A2^+$, and ROT diets showed a significantly ($p < 0.05$) higher SGR% (9.46 ± 0.31, 10.45 ± 0.31, and 9.77 ± 0.30% for the $A1^+$, $A2^+$, and ROT groups, respectively) compared to those fed CTRL and PG diets. Finally, the A^- group (9.22 ± 0.38%) did not evidence significant differences compared to the CTRL group.

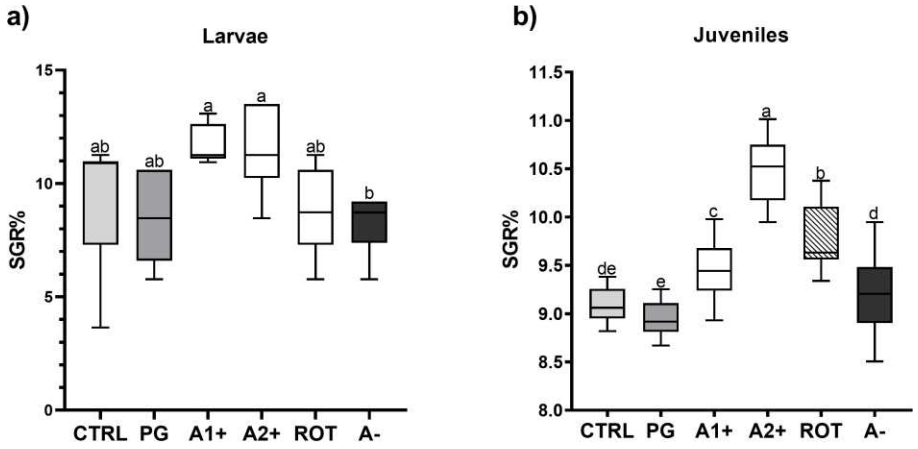

Figure 1. Specific growth rate (% day $^{-1}$) of zebrafish (**a**) larvae and (**b**) juveniles fed the experimental diets. Boxplots show minimum and maximum (whiskers), first quartile, median and third quartile (box). $^{a-e}$ Different letters indicate statistically significant differences among the experimental groups ($p < 0.05$).

Feed Intake

Figure 2 shows the results of the feed intake experiment conducted on zebrafish juveniles fed the experimental diets. No significant differences were detected between CTRL and PG groups. Fish fed the A1$^+$ and A2$^+$ diets showed a higher feed intake respect to both CTRL and PG groups. Finally, no significant differences were evident among CTRL, PG, ROT, and A$^-$ groups.

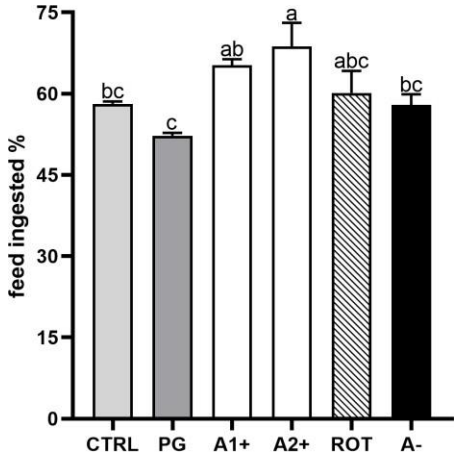

Figure 2. Percentage of feed ingested after 15-min administration of experimental diets in zebrafish juveniles. Results are expressed as mean + SD (n = 3). $^{a-c}$ Different letters indicate statistically significant differences among the experimental groups ($p < 0.05$).

Histology

Considering intestine, no significant alterations in the tissue's architecture or signs of inflammation were evident in either larvae or juveniles belonging to all the experimental groups (Figure 3a,b and Figure 3d,e, respectively). In addition, as reported in Table 2, no significant differences in all the histopathological indexes analyzed were detected in either larvae or juveniles.

As regards liver, both larvae and juveniles belonging to all the experimental groups showed a modestly fat liver parenchyma, with a diffuse presence of hepatocytes with cytoplasm filled with fat (Figure 3c,f). These results were consistent with the statistical quantification of the fat fraction percentage calculated on liver sections obtained from both larvae and juveniles, which did not evidence significant differences among the experimental groups (larvae: 47.6 ± 1.6, 49.9 ± 4.3, 42.6 ± 1.0; 51.9 ± 3.5, 46.1 ± 4.1, and 42.0 ± 5.9 for the CTRL, PG, $A1^+$, $A2^+$, ROT, and A^- groups, respectively; juveniles: 52.0 ± 4.3, 54.2 ± 4.3, 54.6 ± 4.1, 53.4 ± 3.6, 54.6 ± 1.0, and 54.7 ± 2.6 for the CTRL, PG, $A1^+$, $A2^+$, ROT, and A^- groups, respectively).

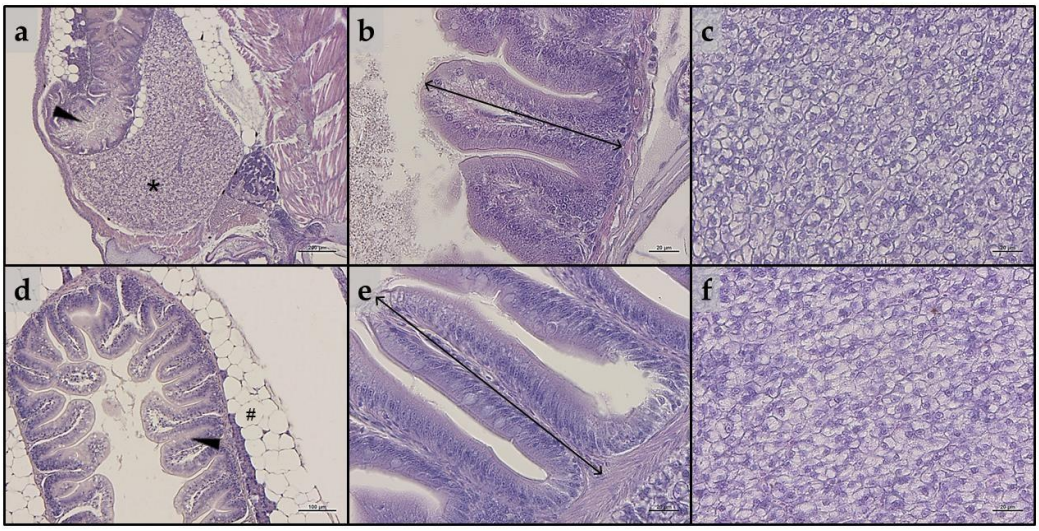

Figure 3. Example of histomorphology of intestine and liver parenchyma of zebrafish (**a**-**c**) larvae and (**d**-**f**) juveniles from the present study. (**a**) representative section of liver (*) and intestine (arrowhead) of a whole-embedded zebrafish larva fed CTRL diet; (**b**) details on mucosal folds of intestine from a zebrafish larva fed PG diet (double-headed arrow indicate mucosal folds height); (**c**) liver parenchyma from a zebrafish larva fed $A1^+$ diet; (**d**) section of intestine (arrowhead) from a zebrafish juvenile fed $A2^+$ diet (# indicate perivisceral adipose tissue); (**e**) details on mucosal folds of intestine from a zebrafish juvenile fed ROT diet (double-headed arrow indicate mucosal folds height); (**f**) liver parenchyma from a zebrafish juvenile fed A^- diet. Scale bars: a = 200 µm; b,c,e,f = 20 µm; d = 100 µm.

Table 2. Histological indexes measured in the intestine of larvae and juveniles fed the experimental diets.

	CTRL	PG	$A1^+$	$A2^+$	ROT	A^-
Larvae						
Mucosal folds height	80.7 ± 0.6	93.0 ± 7.2	84.5 ± 5.5	95.4 ± 7.5	92.5 ± 7.2	75.0 ± 4.5
Inflammatory influx	+	+	+	+	+	+
Mucosal folds fusion	+	+	+	+	+	+
Juveniles						
Mucosal folds height	166.2 ± 23.1	168.0 ± 23.0	160.0 ± 12.0	175.3 ± 26.2	162.4 ± 12.7	164.7 ± 25.5
Inflammatory influx	+	+	+	+	+	+
Mucosal folds fusion	+	+	+	+	+	+

Data of mucosal folds height are reported as mean ± SD (n = 15). No significant differences were detected among experimental groups ($p > 0.05$).

Real-Time PCR Results

Growth. Considering larvae, no significant differences were observed among the experimental groups in both *igf1* and *mstnb* gene expression (Figure 4a,b).

As regards juveniles, no significant differences for either of the genes analyzed were detected between the CTRL and PG groups. However, a significant ($p < 0.05$) *igf1* upregulation and a significant ($p < 0.05$) *mstnb* downregulation were observed in the $A1^+$ and $A2^+$ groups compared to the all the other experimental groups, which did not show significant differences among them for either of the genes analyzed (Figure 4c,d).

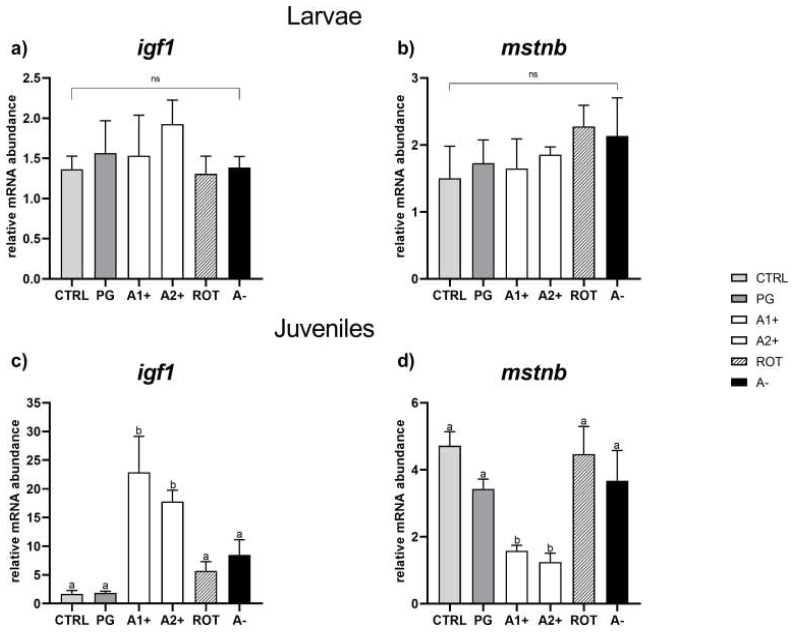

Figure 4. Relative mRNA abundance of genes involved in growth analysed in whole larvae or in liver samples from juveniles. (**a**) *igf1* and (**b**) *mstnb* in larvae; (**c**) *igf1* and (**d**) *mstnb* in juveniles. Results are expressed as mean + SD (n = 5). [a-b] Different letters denote statistically significant differences among the experimental groups; ns, no significant differences.

Appetite. As regards larvae, no significant differences were detected among all the experimental groups for any of the genes analyzed (Figure 5a–c).

Considering juveniles, no significant differences were evident in *ghrl* expression between the CTRL and PG groups. Fish fed $A1^+$, $A2^+$, and ROT diets showed a significantly ($p < 0.05$) lower *ghrl* expression compared to those fed the CTRL one. Finally, the A^- group did not show significant differences in terms of *ghrl* expression compared to both the CTRL and PG groups (Figure 5d).

Considering *npy* relative gene expression (Figure 5e), the PG group showed a significantly ($p < 0.05$) lower value compared to the CTRL one. The $A1^+$ group was characterized by a significant ($p < 0.05$) gene expression downregulation, while the $A2^+$ group showed a significant upregulation compared to the CTRL group. Finally, both the ROT and A^- groups were characterized by a significant ($p < 0.05$) *npy* downregulation compared to the CTRL one.

Ultimately, as regards the *lepa* relative gene expression (Figure 5f), no significant differences were detected between the CTRL and PG groups. Fish fed the $A1^+$, $A2^+$, and ROT diets evidenced a significant ($p < 0.05$)

lepa upregulation compared to those fed the CTRL and PG ones. The A^- group showed a significantly ($p < 0.05$) higher gene expression compared to the PG group, while no significant differences were detected compared to the CTRL group.

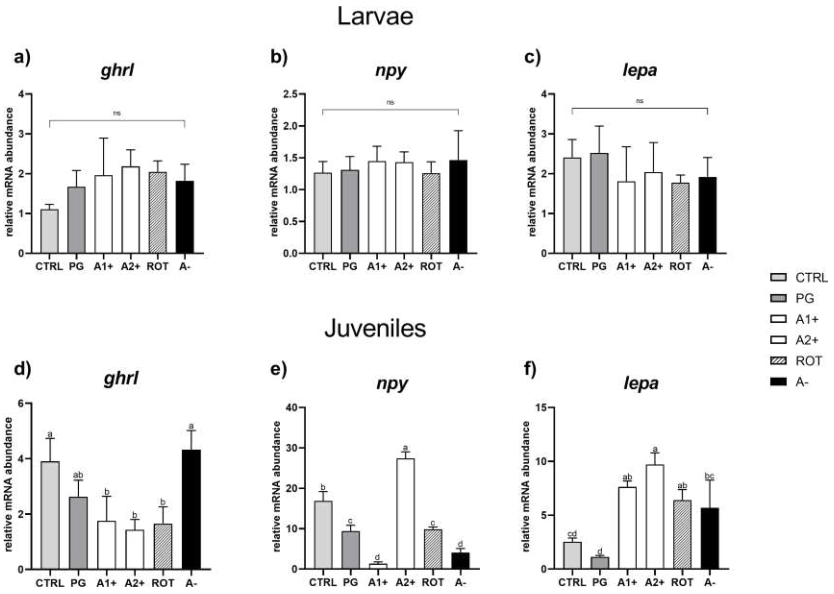

Figure 5. Relative mRNA abundance of genes involved in appetite regulation analyzed in whole larvae or in intestine (*ghrl*), brain (*npy*), and liver (*lepa*) samples from juveniles. (**a**) *ghrl*, (**b**) *npy*, and (**c**) *lepa* in larvae; (**d**) *ghrl*, (**e**) *npy*, and (**f**) *lepa* in juveniles. Results are expressed as mean + SD (n = 5). $^{a-d}$ Different letters denote statistically significant differences among the experimental groups; ns, no significant differences.

Reward system. As regards the larval stage, no significant differences were detected among the experimental groups for all the genes analyzed (Figure 6a–c).

As regards the juvenile stage, CTRL and PG groups did not show significant ($p < 0.05$) differences in *drd1b* gene expression (Figure 6d). The $A1^+$ group was characterized by a significant ($p < 0.05$) *drd1b* downregulation, while the $A2^+$ group showed a significant ($p < 0.05$) *drd1b* upregulation compared to CTRL group. In addition, the ROT and A^- groups did not evidence significant differences compared to the CTRL and PG ones.

Considering the *drd2a* relative gene expression (Figure 6e), the PG group showed a significant ($p < 0.05$) upregulation compared to the CTRL one. The $A1^+$ group did not show significant differences compared to CTRL, while the $A2^+$ and ROT groups showed a significant ($p < 0.05$) *drd2a* upregulation compared to both the CTRL and PG ones. In addition, no significant differences in terms of *drd2a* relative gene expression were observed between the CTRL and A^- groups.

Finally, considering *drd3* relative gene expression (Figure 6f), the PG group showed a significant ($p < 0.05$) upregulation compared to CTRL. No significant differences were observed between the $A1^-$ and CTRL groups, while the $A2^+$ and ROT groups showed a significant ($p < 0.05$) *drd3* upregulation compared to both the CTRL and PG ones. In addition, the A^- group did not show significant differences compared to CTRL.

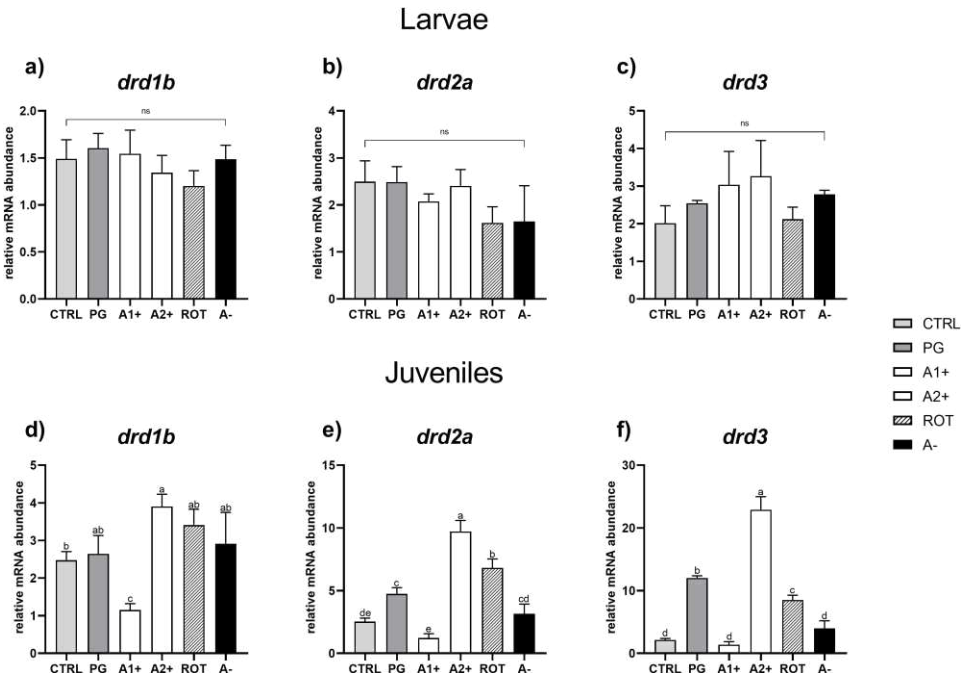

Figure 6. Relative mRNA abundance of genes involved in reward system analyzed in whole larvae or in brain samples from juveniles. (**a**) *drd1b*, (**b**) *drd2a*, and (**c**) *drd3* in larvae; (**d**) *drd1b*, (**e**) *drd2a*, and (**f**) *drd3* in juveniles. Results are expressed as mean + SD (n = 5). [a-e] Different letters denote statistically significant differences among the experimental groups; ns, no significant differences.

Immune and stress response. No significant differences were detected in the expression of genes involved in immune (*il1b*, *il10*, and *litaf*) and stress (*nr3c1*) response in both larvae (Figure 7a–d) and juveniles (Figure 7e–h).

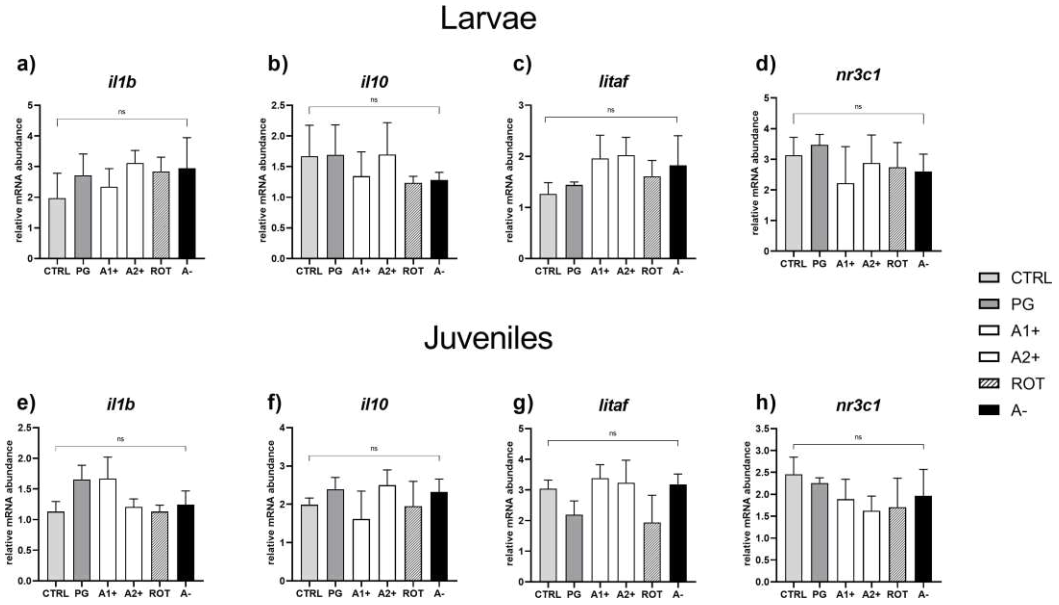

Figure 7. Relative mRNA abundance of genes involved in immune and stress response analyzed in whole larvae or intestine (immune response: *il1b*, *il10*, and *litaf*) and liver (stress response: *nr3c1*) samples from juveniles. (**a**) *il1b*, (**b**) *il10*, (**c**) *litaf*, and (**d**) *nr3c1* in larvae; (**e**) *il1b*, (**f**) *il10*, (**g**) *litaf*, and (**h**) *nr3c1* in juveniles. Results are expressed as mean + SD (n = 5). ns, no significant differences.

Total Lipid Content

The analyzed larvae contained between 0.94 g (in CTRL group) and 1.82 g (in ROT group) of total lipids in 100 g of larvae pool. The total lipid content found in 100 g of larvae was 1.16, 1.38, 1.51, and 1.52 g in PG, $A2^+$, A^-, and $A1^+$, respectively. For juveniles, as reported in Figure 8, the PG group was characterized by significantly ($p < 0.05$) lower total lipid content compared to CTRL. All the groups fed the flavor-added diets ($A1^+$, $A2^+$, ROT, and A^-) showed a significantly higher total lipid content compared to both the CTRL and PG groups.

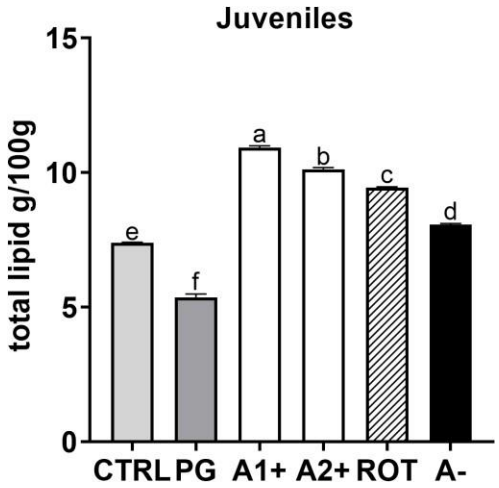

Figure 8. Total lipid content (g/100 g) of zebrafish juveniles. [a-f] Different letters show statistically significant differences among experimental groups ($p < 0.05$). Values are reported as mean + SD (n = 3).

Discussion

Recently, in modern aquafeed formulation, traditional protein sources like FM have been replaced by more sustainable ones, often affecting feed digestibility and palatability [57], resulting in aquafeed wastage, and economic and environmental issues [15,33]. Since the use of natural feed attractants can only partially mitigate this problem, it is crucial to identify and test the efficiency of a set of new feed attractants intended for fish diets [10,19]. In light of this, the present study assessed the potential role of synthetic flavors as feed attractants during the early development of zebrafish. Specifically, the present study tested two positive/attractive synthetic flavors (F25, which showed a short-lasting attractive effect, and F35, which showed a long-lasting attractive effect) and a repulsive synthetic flavor, all of them composed by the same basic solvent (Propylene Glycol—PG). In the present study, both zebrafish larvae and juveniles fed the PG diet generally showed comparable results to those fed the CTRL diet, underlying, as already reported in the literature [58,59], the absence of effects related to the basic solvent used. In addition, as expected, all the tested synthetic flavors had no adverse effects on the overall welfare of either larvae or juveniles. In fact, no alterations in tissue architecture and histopathological indexes, or signs of inflammations were evident in liver and intestine and no significant differences in the expression of immune-related (*il1b*, *il10*, and *litaf*) and of stress-related (*nr3c1*)

markers were detected at either developmental stage. This aspect is very important since when new aquafeed ingredients or additives are used, gut welfare [60] and stress response [61] should always be carefully analyzed. Considering all the other results obtained in the present study, a different scenario was observed in zebrafish larvae and juveniles. As regards the larval stage, most of the analyses performed did not show significant differences among the experimental groups, suggesting that the exposure time (21 dpf) was probably too short. Additionally, it should also be pointed out that fish larval stages are characterized by very high growth rates and quick and deep morphological modifications [62,63] which could have reduced differences among groups. However, it should be noted that besides the less pronounced growth differences of the larval stage compared to the juvenile stage, larvae fed $A1^+$ and $A2^+$ diets showed a significantly higher SGR% compared to the repulsive A^- group, underlining that the behavioral test properly selected the flavors. As already mentioned, the juvenile stage provided more marked responses in relation to the tested flavors, giving important insights about their potential role as novel feed attractants. Specifically, their attractive role was confirmed by the feed intake experiment and supported by the molecular analysis. The highest feed intake obtained within 15 min experiment was observed in both zebrafish juveniles fed $A1^+$ and $A2^+$ diets, a result that might be of interest for the aquafeed production sector to avoid feed waste. On this regard, it has to be mentioned that the fish feeding behavior is moved by the appetite stimulus that results from the interplay between orexigenic and anorexigenic signals, both produced at the peripheral-gut and central-brain levels [23]. Within this crosstalk, ghrelin is mainly produced at the gut level, playing an essential role in stimulating feeding activity [64]. Regarding the present study, the significant *ghrl* downregulation observed in zebrafish juveniles fed $A1^+$, $A2^+$, and ROT diets suggested that they were in a more satiated state compared to those from the other groups. These data also fully confirmed the feed intake results at the molecular level [65]. Conversely, nonobvious results were obtained by analyzing the *npy* gene expression at the brain level. NPY, besides being expressed also in other tissues like spleen, kidney, and smooth muscle [66,67], interacts with a number of different appetite signals such as corticotropin-releasing factor (CRF), cortisol, CART, orexins, and galanin, suggesting that the NPY response is not exclusively dependent from the ghrelin signal [68]. In addition, NPY is involved in several other physiological functions in fish, like circadian rhythmicity, cardiovascular activity, psychomotor activity, and sexual behavior, which could have affected the present results [69–71]. Differently, considering the expression of the anorexigenic signal *lepa*, a significant upregulation, in accord with *ghrl* gene expression, was observed in zebrafish juveniles fed $A1^+$, $A2^+$, and ROT diets, further supporting their more satiated state. Leptin expression is known to be a strong indicator of fish energy reserve availability, being directly related to the amount of adipose tissue and being involved in the mobilization of the fat stored in the liver [22–24]. Despite the absence of significant differences in the hepatic lipid accumulation among the experimental groups analyzed, fish fed $A1^+$, $A2^+$, and ROT diets showed a higher lipid content with respect to the control group, as confirmed by the total lipid analysis. Taken together, the above-described results suggest that both attractive flavors used in the present study promoted a higher feed intake by the $A1^+$ and $A2^+$ groups, which in turn determined a more satiated state of these fish, a higher lipid storage, and a higher growth, which was also confirmed by the molecular analysis. It is thus suggested that diet palatability was improved by adding the two positive flavors selected in this study, especially considering the fact that feed's smell and taste are

hedonic properties able to activate the pleasure sensation during feed ingestion [20]. In this regard, it is well known that attractive and highly palatable feed positively influences appetite regulation and feeding behavior in vertebrates through the activation of the brain reward system [27]. Within this system, the neurotransmitter dopamine and its related receptors are the main actors in promoting feed motivation [72]. Particularly, in the present study, the expression of three different dopamine receptors—Drd1b, Drd2a, and Drd3—were analyzed. The Drd1b is the homolog of the mammalian D1-like receptor type, while the Drd2a and Drd3 are homologous to the mammalian D2-like receptor type [73]. D1 and D2 receptors constitute two structurally and functionally different classes of dopamine receptors, both strongly and positively related to feed intake and feed motivation [74,75]. However, the administration of highly palatable feed over a long-term period of time can contribute to the development of reward system hyposensitivity through the reduction in the expression of the dopamine receptor D2 [75,76]. This hyposensitivity condition seems to induce a compulsive eating behavior, related to an incapacity to achieve the reward expected from the feed [77]. This mechanism has been demonstrated in mammals, but it has been poorly investigated in fish. In the present study, both the attractive diets did not negatively affect the fish dopaminergic activity, with the most interesting results about this aspect observed during the juvenile stage. Zebrafish juveniles fed the $A2^+$ (including the long-lasting F35 flavor) and, in a lesser extent, ROT diets showed a higher expression of all the dopamine receptors analyzed, highlighting a stronger dopaminergic activity compared to the CTRL group and suggesting that the reward expected from feed was maintained throughout the whole experiment. Conversely, $A1^+$ group juveniles (including the F25 short-lasting flavor) showed a dopaminergic activity comparable to CTRL, suggesting that this flavor had less effect than the $A2^+$ one on feeding motivation. As regards the ROT group, which was conceptualized to understand if changing positive flavor administration during the experiment was a good strategy to avoid adaptation to the same *stimulus* and enhancing fish feeding response, it should be pointed out that the results obtained in the present study indicated that this was not a valuable strategy. The administration of a long-lasting attractive flavor as F35, interspersed with a short-lasting one as F25, reduced the overall effectiveness of the feeding scheme, probably because F25 lost its effectiveness as feed attractant over the trial, as confirmed by molecular analysis of dopaminergic activity. Finally, the repulsive flavor used in the present study led to results that were comparable to the CTRL group, suggesting a possible adaptation by the fish to the flavor or a potential interaction between the flavor and the feed odor. However, because of the preliminary results obtained through the behavioral test, this was used as negative control.

Conclusions

All the synthetic flavors tested did not affect the overall health of fish and, for the positive ones, confirmed their attractive role, as already demonstrated by the preliminary behavioral trial. The provision of diets containing attractive flavors resulted in an increased growth rate in both larvae and juveniles. Due to the longer exposure time, results obtained from the juvenile stage provided a clearer picture of the fish responses. In fact, zebrafish juveniles fed $A1^+$ and $A2^+$ diets ($A1^+$ cheese odor made by mixing Propylene Glycol (PG) with aromatic chemicals trimethyamine, 2-acetylpyrazine, 2-acetylpyridine, and dimethyl sulfide; and $A2^+$ caramel odor, made of PG mixed with the aromatic chemicals vanillin, maltol, cyclotene, acetoin, butyric acid, and capric acid with traces of both gammaoctalactone and gamma-esalactone, respectively) showed higher appetite stimulus, feed ingestion, and growth. However, the modulation of the brain reward system suggested the selection of the $A2^+$ diet (and thus the F35 flavor) as the most valuable solution for maintaining its long-lasting effect over the whole experimental period. As regards the A^- group (coconut odor, made by mixing PG with the aromatic chemicals gamma-eptalactone, gamma-nonalactone, delta-esalactone, and vanillin with traces of both delta-octalactone and maltol), the negative effect evidenced during the behavioral test conducted on the flavor exclusively was mitigated by mixing the flavor with the diet. In conclusion, the present study provides important results about the possible use of attractive synthetic flavors for the aquafeed production, opening new sustainable and more economically valuable opportunities for the aquaculture sector. Further research has to be developed, testing the same flavors on adult fish with an emphasis on reproduction, since fish life stages may have behavioral differences related to their developmental stages.

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Chapter 3

The promising role of synthetic flavors in advancing fish feeding strategies: a focus on adult female zebrafish (*Danio rerio*) growth, welfare, appetite, and reproductive performances

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Abstract

The present study was aimed to test over a six-month period different synthetic flavors in zebrafish (*Danio rerio*) as experimental model. Specifically, two attractive and one repulsive synthetic flavor were added (1% *w*/*w*) to a specific zebrafish diet which was administered to the fish during the whole life cycle (from larvae to adults), to evaluate their physiological responses, giving emphasis on fish welfare, feed intake, growth, reward mechanisms, and reproductive performances. Fish welfare was not affected by all tested flavors, while both attractive flavors promoted fish feed ingestion and growth. Results were supported by both molecular and immunohistochemical analyses on appetite-regulating neurohormonal signals, along with the influence of the feed hedonic properties induced by the brain reward sensation, as demonstrated by the dopamine receptor gene expression. Finally, the present study demonstrated that a higher feed intake also had positive implications on fish reproductive performances suggesting a promising role of synthetic flavors for the aquaculture industry. In conclusion, the results highlighted the potential of synthetic flavors to improve fish feeding strategies by providing a consistent and effective alternative to traditional stimulants, thereby reducing dependence on natural sources.

Introduction

In recent years, promising progress in validating the use of alternative and cheaper aquafeed ingredients, especially respect to fishmeal, has been made. However, aquafeeds still represent a significant portion of production costs in aquaculture. Novel ingredients encompass plant-derived meals, processed animal proteins obtained by animal by-products and insects, and microbial biomasses [1–7]. Despite their promising features, they can present a poor palatability with consequent negative implications for fish production and farm economics [8–10]. Thereby, reducing feed losses through improved feeding practices is becoming crucial [11,12]. In this regard, additive ingredients typically derived from natural sources, are regularly included in aquafeeds to improve palatability [13,14]. These ingredients, listed as feed attractants, are crucial in fish diets formulation to enhance feed efficiency [15], as by including feeding stimulants in the diet, fish are more likely to seek and consume the feed faster. In fact, these attractants stimulate the fish's sensory organs, as taste and olfaction, triggering feeding behavior through the release of chemical stimuli in the water [16]. A continuous exposure to different odors in a mutable aquatic environment requires a highly dynamic olfactory system in teleost, that exhibits widespread plasticity of the olfactory sensory neurons (OSNs) to adapt continuously the response to odorants [17]. As a result, feed attractants can improve feed intake, ultimately contributing to fish growth and overall performance in aquaculture operations.

An effective application of feed attractants should simultaneously have a positive impact on fish feed intake and on parameters related to its regulation, such as peripheral and hypothalamic appetite-regulating hormones [18]. In teleost, appetite control and energy balance, at the central-hypothalamus level, are integrated by peripheral endocrine signals via a complex interaction, including orexigenic (neuropeptide Y, ghrelin) [19,20] and, anorexigenic (leptin) [18,21,22] hormones. In addition, the expression of neurohormonal signals in the fish’s olfactory system are known to influence the olfactory sensitivity via a nutritional state-dependent mechanism, reflecting the prevailing energy status in the brain [23,24].

Along with the homeostatic regulation, hedonic and motivational neurocircuits are activated during feeding, involving monoaminergic systems triggered by the fish taste system [25,26]. The mesolimbic dopamine system plays a crucial role in feeding behavior by being closely linked to the reward sensation experienced during the consumption of palatable feed, promoting the desire to eat [18,26].

The currently employed feed attractants are mostly represented by marine extracts (e.g., meal derived from mollusks and crustaceans), further posing unsustainability issues [27]. In addition, the attractiveness of these substances depends on their natural origin, resulting in inconsistent quality of the raw materials, different availability, freshness, and diverse manufacturing practices applied [28,29]. Differently, specific compounds like L-amino acids, small chain peptides, betaine, and nucleotides are potent attractants for fish and are currently used as attractive alternative substances to the traditional feed attractants [30,31]. However, their application presents limitations and disadvantages since their concentration and interaction with other diet’s components can affect the overall performance of the feed [32]. Moreover, the stimulatory effect varies depending on the fish species and life cycle stage considered, while optimal dosage for the different species is

still poorly investigated in available scientific literature [31,32].

Synthetic flavors are emerging as a novel and effective alternatives to traditional feed attractants. Their synthetic origin and standardized production processes can solve the drawbacks related to the use of marine extracts as feed attractants, in terms of: (i) sustainability, reducing the utilization of marine resources for aquafeed production; (ii) attractiveness, reducing the variability related to the inconsistent origin and quality of the raw materials. Particularly, Conti et al. [33] recently demonstrated the efficiency of a number of synthetic flavors, produced through standardized processes, as feed attractants in promoting the appetite stimulus, feed ingestion, and growth during zebrafish (*Danio rerio*) early development (from larvae to juveniles).

Monitoring the long-term effects of synthetic feed attractants is crucial for the aquaculture sector, especially considering that fish production involves various life-cycle stages. Zebrafish represents a widely accepted model organism for preliminary nutrition studies useful to the aquaculture sector [34,35] and many novel aquafeed ingredients have primarily been tested on this experimental model [36–39]. Additionally, the well-established knowledge on zebrafish oocyte development and maturation, and the relative short time to reach sexual maturity make this species ideal to monitor the potential effects of synthetic flavors also on its reproductive performances [40,41]. In fact, since the reproductive system is well conserved across all fish species [42], zebrafish is a useful model for a comprehensive understanding of mechanisms involved in broodstock reproduction [34,35].

Furthermore, the reproductive phase is one of the most important stages of fish production, since breeders are responsible for offspring production. In this context, females reproduction is of great interest since it is particularly energy demanding to sustain oocyte maturation through the proper allocation of macromolecules in developing oocytes [43]. Uptake of vitellogenin from maternal liver and its conversion into yolk protein is the major mechanism for oocyte development [44]. Vitellogenin gene expression is therefore a reliable molecular marker for assessing fish reproduction. In particular, the zebrafish *vtg* gene repertoire is characterized by five (*vtg1*, *vtg4*, *vtg5*, *vtg6*, and *vtg7*) type I, two (*vtg2* and *vtg8*) type II, and one (*vtg3*) type III vtg genes [45]. Notably, among type I vitellogenins, *vtg1* is the predominant form, while *vtg2* is the most abundant among type II Vtgs, according to Yilmaz et al. [45]. The vitellogenin allocation in oocytes establishes the transition from oocytes at previtellogenic stage (defined as I and II) to ones at vitellogenic stage (defined as III onward) [40,41].

Oogenesis can be affected by several stressors including food availability, ingestion, nutritional composition, and assimilation that should be studied in a deeper way to assess the suitability of a diet to correctly sustain fish reproduction [46–48].

In this context, the present study aimed to test the effects of different synthetic flavors during a six-month feeding trial on zebrafish (from larvae to adults) to assess their potential role as feed attractants, giving emphasis on fish feed intake, welfare (defined as the aptitude of an animal to live a natural life and show its natural behavior, while maintaining good health in its environment), feed reward mechanisms, and reproductive performances.

Methods

Ethics

The experimental procedures were approved (n° 2-1/12/22) by the Ethics Committee of the Marche Polytechnic University (Ancona, Italy) and the Italian Ministry of Health (Aut N° 453/2023-PR), and conducted in accordance with the Italian legislation on experimental animals. An anaesthetic (MS222, 1 g/L for fish euthanasia; Merck KGaA, Darmstad, Germany), was used to minimize the suffering of the animals.

Synthetic flavors and production of experimental diets

The three synthetic flavors used for the present study were produced (To Be Pharma S.r.l., S. Egidio alla Vibrata, Teramo, Italy) and selected according to Conti et al. [33]. Briefly, each synthetic flavor consisted of functional solvent, 1,2-propanediol (propylene glycol; PG) in which different flavoring chemicals were dissolved. Both PG and flavoring molecules have been widely considered as safe for use by both the Flavor and Extracts Manufacturers Association and the Food and Drug Administration (Agency for Toxic Substances and Disease Registry 2007).

In this study, two attractive flavors, namely F25 (cheese flavor) and F35 (caramel flavor), and one repulsive flavor, named F32- (coconut flavor), were produced in compliance with the current sector-specific legislation, specifically Regulation (EC) No 1334/2008 and 1333/2008. Additionally, technical data sheet and the Material Safety Data Sheet (MSDS) confirm the safety of these flavors for both humans and animals. This information ensures that all the flavors used in the study meet regulatory standards and are considered generally safe.

As a control diet (CTRL), a commercial zebrafish diet (Zebrafeed; Sparos LDA, Olhão, Portugal) was selected. Then, four experimental diets were prepared starting from the CTRL diet as follows: (i) PG diet, obtained by adding PG to the CTRL diet; (ii) F25 diet, obtained by adding flavor to the CTRL diet; (iii) F35 diet, obtained by adding F35 flavor to the CTRL diet; (iv) F32- diet, obtained by adding F32- flavor to the CTRL diet. All the synthetic flavors and PG were added to the CTRL diet at a concentration of 1% (w/w).

Experimental design

Zebrafish embryos were obtained from the University AB strain broodstock, collected, and maintained (Tecniplast system; Varese, Italy) at: pH 7 ± 0.1, 28 ± 0.5 °C, concentrations of ammonia and nitrite < 0.01 mg/L, nitrate concentration < 10 mg/L, and 12L/12Dof photoperiod. After embryo development (48 hours), 9000 alive embryos were collected (selecting them under a stereomicroscope) and randomly divided into the six experimental groups (3 tanks per experimental group with 500 embryos/tank). and fed according to Conti et al. [33]: (i) CTRL group; (ii) PG group, zebrafish fed PG diet; (iii) F25 group, zebrafish fed F25 diet; (iv) F35 group, zebrafish fed F35 diet; (v) F32- group, zebrafish fed F32- diet (negative control); (vi) rotation (ROT) group, zebrafish fed F25 and F35 diets, each one singularly administered in a weekly rotation scheme. The inclusion of the ROT group was intended to avoid the possible adaptation of fish olfactory receptors in response to a repeated stimulation, as reported by Zufall & Leinders-Zufall [49]. At the beginning of the experiment, 18 tanks (20 L, 3 tanks per experimental group with 500 fish per tank, 1500 per dietary group)

with the same broodstock's water conditions, were used to rear zebrafish during the larval stage, with filtration and light set up as described in Olivotto et al. [50]. After 30 days post fertilization (dpf), fish from each tank were gently moved to other tanks (100 L, 3 tanks per experimental group) provided with mechanical and biological filtration (Panaque, Capranica, Italy).

From 5 dpf to the end of the trial (180 dpf), zebrafish were fed the experimental diets(3% body weight, divided in two daily feedings, according to Rashidian et al. [51]).

During the feeding trial, feed particle sizes were: <100 μm from 5 to 15 dpf, 101–200 μm from 16 to 30 dpf, 201–400 μm from 31 to 60 dpf, and 401-600 μm from 61 to 180 dpf. Additionally, *Brachionus plicatilis* rotifers (5 individuals/mL) were provided to zebrafish larvae of all the experimental groups from 5 to 10 dpf twice a day, according to Zarantoniello et al. [52]. During the trial, tanks were daily cleaned, and dead specimens, if present, were collected and counted.

At the end of the trial, 144 zebrafish females were sampled, after a lethal dose of MS222 (1 g/L, Merck KGaA), and whole head, brain, liver, intestine, and ovary were sampled and properly stored for the laboratory analysis. Particularly: (i) liver, intestine and ovary from 5 female zebrafish per tank were fixed in Bouin's solution for histological analysis; (ii) whole head from 5 female zebrafish per tank were fixed in formalin for himmunohistochemistry; (iii) liver, intestine and brain from 3 female zebrafish per tank were stored at –80 °C for molecular analysis. Further information are detailed in the dedicated paragraphs.

Biometry and reproductive parameters

At the end of the trial (6 months), 20 fish from each tank (60 per experimental group) were weighted with a OHAUS Explorer analytical balance (Greifensee, Switzerland), accuracy: 0.1 mg.

Additionally, ovaries were individually weighted to calculate gonadosomatic (GSI) index applying the following formula:

$$GSI = [(\text{ovary weight}/\text{whole body weight}) \times 100] \quad (1)$$

Survival rate was calculated (at 6 months) by subtracting the dead fish (daily calculated over the whole experimental period) to the initial number of fish per tank.

Feed Intake

The amount of feed ingested by six-month old zebrafish was evaluated during a 10-day period, according to Conti et al. [33]. During the experiment, a pre-weighed quantity of feed was administered, corresponding to 3% of their body weight, to each tank belonging to the different experimental groups. The eventual uneaten feed, in each tank, was recovered 15 min post-administration of the experimental diets by siphoning, and then oven-dried overnight at 40 °C for quantification. The 15-minute duration was chosen in accordance with Conti et al. [33], along with the fact that an effective feed attractant must stimulate a rapid ingestion of the feed within the first minutes of administration [53].

Egg collection and hatching rate

For a 10-day period, six-month old zebrafish (7 males and 5 females from each experimental tank, in triplicate) were daily randomly collected and transferred to breeding tanks (Tecniplast System; 9 breeding tanks per experimental group), in accordance with Randazzo et al. [54]. During both the acclimatizing (which lasted one week) and breeding periods, fish were fed experimental diets and maintained under the same conditions of the experimental tanks. From each breeding tank (30 minutes after the onset of light), eggs were daily collected and counted under a stereomicroscope (Leica Wild M3B; Leica Microsystems) for the evaluation of daily spawned eggs. Subsequently, only fertilized eggs that had a well-developed blastodisc at 3 h post-fertilization were properly stored and used for the hatching test. One hundred and fifty embryos from each experimental group (in triplicate) were randomly selected and transferred to Petri dishes (50 embryos per dish) with E3 medium, prepared as described in Randazzo et al. [54]. Embryos were kept in an incubator at 28 °C for 5 dpf, to evaluate. hatching rate Hatching rate was calculated as hatched eggs/total fertilized eggs × 100%. The hatched larvae were finally counted (number of alive fish at 5 days) and hatching rate calculated.

Histological analysis

Livers, intestines and ovaries from 5 female specimens per tank (15 per experimental group), were processed using the procedures described in Randazzo et al. [55].

Three transversal sections per fish (15 intestines per experimental group), collected at 50 μm intervals, were analyzed to evaluate potential alterations in intestinal tract and hepatic parenchyma. Specifically, regarding intestine, analyses were conducted on all the undamaged and non-oblique folds and both measurements and histopathological indexes were applied in accordance with Zarantoniello et al. [52] and using ZEN 2.3 software (Zeiss, Oberkochen). Indexes score were assigned as follows: (i) inflammatory influx: + = scarce lymphocytes infiltration, ++ = moderate lymphocytes infiltration, +++ = diffused lymphocytes infiltration; (ii) mucosal folds fusion: + = 0-3 observations per section, ++ = 3-10 observations per section, +++ = > 10 observations per section.

As regards liver, the fat fraction percentage was quantified on areas with no blood vessels or bile ducts using ImageJ software (setting a homogeneous threshold value) and expressed as percentage of the area occupied by fat on the total hepatic parenchyma (images acquired at 400×).

From each fully sectioned ovary (15 per dietary group), a significant number of histological sections was collected at 300 μm intervals. Subsequently, on stained sections, previtellogenic (classes I and II), class III, class IV, and class V, oocytes were counted, according to their morphological features, as described in Randazzo et al. [54]. Oocyte count was performed by using a Zeiss Axio Imager.A2 (Oberkochen, Germany) microscope and ZEN 2.3 lite Software (Zeiss), marking oocytes in each section to minimize repetition, in accordance with Chemello et al. [2].

Immunohistochemistry analysis

Whole heads from 5 adult zebrafish per tank (in triplicate, 15 per experimental group) were collected at the end of the experiment. Heads were fixed in formalin solution (Bio Optica). Paraffin embedding step was performed according to Randazzo et al. [55]. Sections of 5 µm in thickness were cut from the solidified paraffin blocks using a microtome (Leica RM RTS). The sensory area of zebrafish olfactory epithelium was identified as described in Villamayor et al. [56]. In particular, the typical olfactory rosette was identified at its maximum expression when divided into two symmetrical zones by the presence of a wide central stalk, that serves to support the lamellae. Lamellae, located in the central and medial region of the rosette, are composed of a continuous sensory area, as well as a lateral non-sensory epithelium. The sensory area comprises a characteristic pseudostratified columnar epithelium formed primarily by olfactory sensory neurons (OSNs), as well as basal and supporting cells [57–59].

According to Lombó et al. [60], slices were incubated in a buffer containing 10 mM sodium citrate and 0.05% (v/v) Tween at 100 °C for 20 min, to perform an antigen retrieval. The sections were then permeabilized in 1% PBS-T (triton X-100) for 20 min, after washing them with distilled water. One hour blocking was carried out in PBS supplemented with 3% BSA and 0.1% (v/v) Tween. Polyclonal anti-NPY antibody (1:5000; Sigma-Aldrich, Cat. No. N9528, 2018), was used as primary antibody for sections incubation, performed overnight at 4 °C. Subsequently, sections were incubated with a Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488; ab150077, Abcam) at 37 °C for 1 h, after three washing steps with PBS. In the final step, slides were mounted with DAPI-Aqueous Fluoroshield (ab104139, Abcam). The images were acquired in five different sections per sample, using a confocal microscope Nikon A1R, collected at 20 µm intervals. For the qualitatively analysis of the NPY-expressing neurons, found in the sensory area of the lamellae, scores were assigned as follows: + = scarce NPY-expressing cells distribution, ++ = moderate NPY-expressing cells distribution, +++ = diffused NPY-expressing cells distribution.

The specificity of the anti-NPY antibody (Sigma-Aldrich) used in this study was previously demonstrated by Kaniganti et al. [61].

Molecular analyses

Gene expression of target genes in brain, liver, and whole intestine samples from 3 female fish per tank (in triplicate, 9 brains, 9 livers and 9 intestines per group) was measured through real-time PCR. Total RNA extraction and purification, cDNA synthesis, and were performed according to Olivotto et al. [62] and following the manufacturers' protocols. The integrity of RNA was assessed by running 1 µg of total RNA stained with GelRedTM on a 1% agarose gel.

Real-Time quantitative PCR (qPCR) reactions were performed in an iQ5 iCycler thermal cycler (Bio-Rad) on a 96-well plate, following the parameters and reagents reported in Cattaneo et al. [63]. Relative mRNA abundance was used to quantify the gene expression using, as housekeeping genes (two), the ribosomal protein L13 (*rpl13*) and actin-related protein 2/3 complex subunit 1a (*arpc1a*), to standardize the results. The relative quantification of genes involved in fish growth (insulin-like growth factor, *igf1*; myostatin, *mstnb*), appetite regulation (ghrelin, *ghrl*; neuropeptide Y, *npy*; and leptin, *lepa*), brain reward system (dopamine receptor D2a,

drd2a), stress response (glucocorticoid receptor, *nr3c1*), and vitellogenin production (vitellogenin 1, vtg1; vitellogenin 2, *vtg2*; vitellogenin 3; *vtg3*) was performed on samples from target organs. Particularly: (i) *igf1*, *mstnb*, *nr3c1*, *lepa*, *vtg1*, *vtg2*, and *vtg3*, were tested in liver samples; (ii) *ghrl* was tested in intestine samples; (iii) *npy*, and *drd2a* were tested in brain samples. The primers sequences used to evaluate the gene expressions in this study are reported in Table 1. The efficiency was around 90% for all the tested primers, with an R^2 that ranged from 0.995 to 0.998. The fluorescence was monitored at the end of each cycle, and the melting curve analyses revealed one single peak for every product. Two no-template controls (NTCs) were added in each run to guarantee absence of contamination in the Master Mix. Amplification products were sequenced, and homology was verified. The mRNA levels of target genes were calculated using the geometric mean of two stably expressed reference genes, using the Bio-Rad CFX Manager 3.1 software. Gene expression changes among groups are reported as relative mRNA abundance. The qPCR data were processed using iQ5 optical system software version 2.0 (Bio-Rad), including GeneEx Macro iQ5 Conversion and genex Macro iQ5 files.

Table 1. Primer sequences used in the present study, annealing temperature (AT), and NCBI IDs.

Gene	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	AT (°C)	ID
igf1	GGCAAATCTCCACGATCTCTAC	CGGTTTCTCTTGTCTCTCTCAG	53	ZDB-GENE-010607-2
mstnb	GGACTGGACTGCGATGAG	GATGGGTGTGGGGATACTTC	58	ZDB-GENE-990415-165
ghrl	CAGCATGTTTCTGCTCCTGTG	TCTTCTGCCCACTCTTGGTG	58	ZDB-GENE-070622-2
npy	GTCTGCTTGGGGACTCTCAC	CGGGACTCTGTTTCACCAAT	60	ZDB-GENE-980526-438
lepa	CTCCAGTGACGAAGGCAACTT	GGGAAGGAGCCGGAAATGT	60	ZDB-GENE-081001-1
drd2a	TGGTACTCCGGAAAAGACG	ATCGGGATGGGTGCATTTC	58	ZDB-GENE-021119-2
vtg1	GCCAAAAAGCTGGGTAAACA	AGTTCCGTCTGGATTGATGG	59	ZDB-GENE-001201-1
vtg2	TGCCGCATGAAACTTGAATCT	GTTCTTACTGGTGCACAGCC	58	ZDB-GENE-001201-2
vtg3	GGGAAAGGATTCAAGATGTTCAGA	ATTTGCTGATTTCAACTGGGAGAC	58	ZDB-GENE-991019-2
nr3c1	AGACCTTGGTCCCCTTCACT	CGCCTTTAATCATGGGAGAA	58	ZDB-GENE-050522-503
rpl13 (hk)	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG	59	ZDB-GENE-031007-1
arpc1a (hk)	CTGAACATCTCGCCCTTCTC	TAGCCGATCTGCAGACACAC	60	ZDB-GENE-040116-1

Abbreviations: hk, housekeeping genes.

Statistical analyses

Normality (Shapiro–Wilk test) and homoscedasticity (Levene's test) were checked for all data. ANOVA followed by Tukey's multiple comparison post hoc test (software package Prism 8; GraphPad software version 8.0.2, San Diego, CA, USA) was applied to analyze all the data from the different analyses (significance set at $p < 0.05$).

Results

Biometry

As regard survival rate, no significant differences were evident among the experimental groups (87 ± 4; 85 ± 5, 86 ± 4, 85 ± 3, 86 ± 4, and 84 ± 3 % for CTRL, PG, F25, F35, ROT, and F32-, respectively). Figure 1 shows the final body weight (FBW) of specimens fed the experimental diets. CTRL and PG groups did not show significant differences. These groups instead showed a significantly ($p < 0.05$) lower FBW compared to the other groups. In addition, specimens from F35 group showed a significantly ($p < 0.05$) higher FBW compared to all the other experimental groups. Finally, F25 group showed a significantly ($p < 0.05$) higher FBW compared to ROT group.

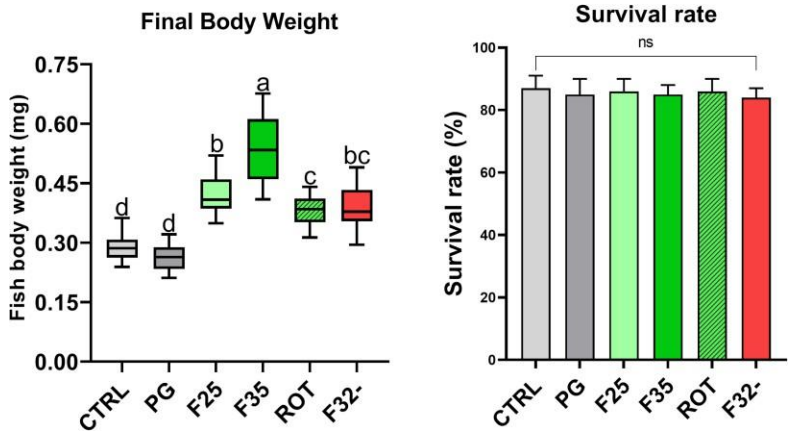

Figure 1. Final body weight (mg) (left) and survival rate (%) (right) of adult zebrafish fed the experimental diets. Boxplot shows minimum and maximum (whiskers), first quartile, median and third quartile (box). [a-d] Different letters indicate statistically significant differences among the experimental groups ($p < 0.05$). Values are presented as mean ± SD (n = 3). ns, no significant differences.

Feed intake

Figure 2 shows the percentages of feed ingested by adult zebrafish fed the experimental diets. No significant differences were detected between the CTRL and PG groups, while both F25 and F35 groups showed a significantly ($p < 0.05$) higher feed intake compared to CTRL, PG and F32- groups. No significant differences were evident among the remaining groups.

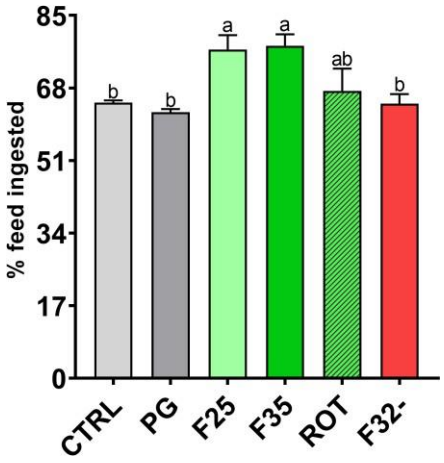

Figure 2. Percentage of feed ingested by adult zebrafish, calculated 15 minutes post dietary administration. Results are expressed as mean + SD (n = 3). [a-b] Different letters indicate statistically significant differences among the experimental groups ($p < 0.05$).

Liver and intestine histomorphology

Regarding intestine and liver of specimens from the different experimental groups, no remarkable alterations in the tissues' morphology or any signs of inflammation were observed (Figure 3). Corroborating this, no significant differences were detected in all histopathological indexes analyzed in the intestine (Table 2). Additionally, a physiological fat accumulation was observed in the hepatic parenchyma of all experimental groups, further supported by the statistical quantification of the fat fraction percentage calculated on liver sections, which showed no significant differences among groups (41.5 ± 6.1, 36.9 ± 1.8, 32.9 ± 10.1, 22.9 ± 6.1, 39.2 ± 2.9, 22.1 ± 3.2%, for CTRL, PG, F25, F35 ROT and F32-, respectively; $p > 0.05$).

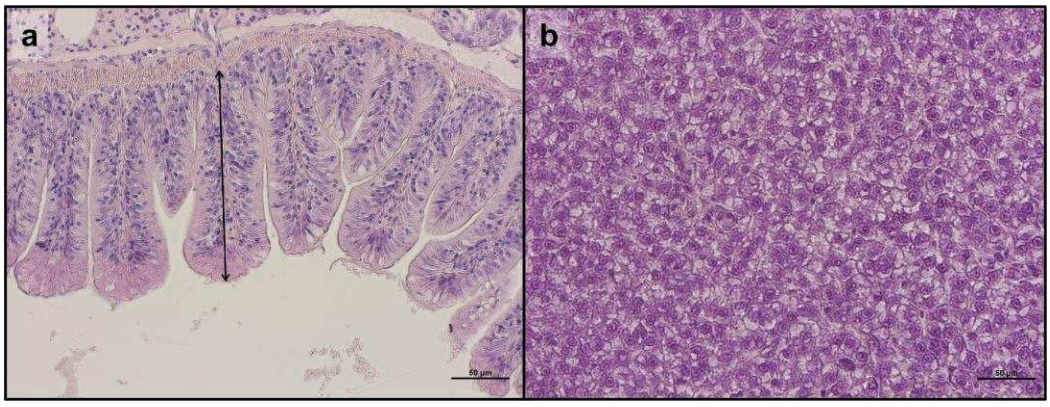

Figure 3. Representative figures of intestine and liver parenchyma of adult zebrafish (a-b) from the present study. (a) details on mucosal folds (double-headed arrow: mucosal folds height); (b) liver parenchyma. Scale bars: 50 µm.

Table 2. Histological indexes measured in the intestine of adult zebrafish fed the experimental diets. Data for intestinal mucosal folds height are reported as mean ± SD (n = 15).

	CTRL	PG	F25	F35	ROT	F32-
Mucosal folds height	197.9 ± 8.6	213.3 ± 41.4	222.8 ± 54.3	180.2 ± 11.1	177.4 ± 16.1	183.5 ± 20.6
Inflammatory influx	+	+	+	+	+	+
Mucosal folds fusion	+	+	+	+	+	+

Real-time PCR

Growth. As regards *igf1*, no significant differences were detected between CTRL and PG groups (Figure 4a). Conversely, F35 and ROT groups showed a significantly ($p < 0.05$) higher expression of *igf1* compared to CTRL, PG and F32- groups (no significant differences among them).

As regards *mstnb*, no significant differences were detected among the different experimental groups (Figure 4b).

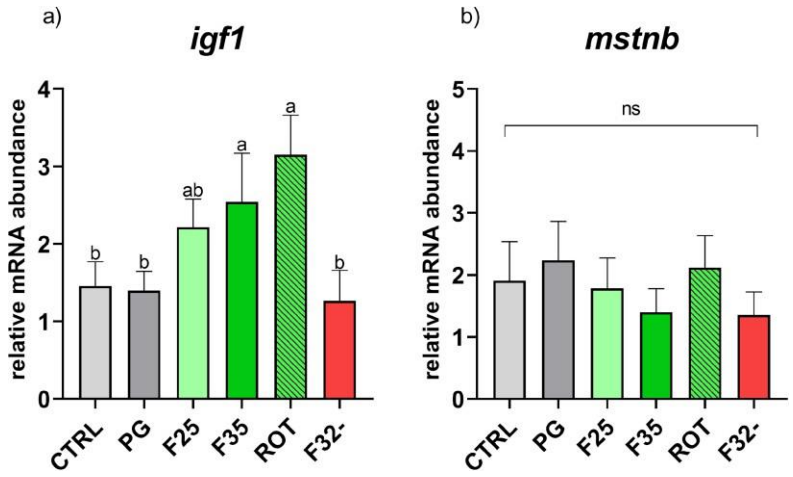

Figure 4. Relative mRNA abundance of genes involved in growth analyzed in liver samples from adult zebrafish. (a) *igf1* and (b) *mstnb*. Results are expressed as mean + SD (n = 5). [a-b] Different letters denote statistically significant differences among the experimental groups; ns, no significant differences.

Appetite. Considering *npy* gene expression (Figure 5a), CTRL and PG groups did not show significant differences among them. Conversely, F25 and F35 groups were characterized by a significantly ($p < 0.05$) lower *npy* expression compared to the other groups.

With respect to *ghrl* gene expression (Figure 5b), fish fed the CTRL, PG and F35 diets did not show significant differences among them while CTRL and F35 showed a significantly ($p < 0.05$) higher *ghrl* expression compared to the other groups. Additionally, F25 group showed a significantly ($p < 0.05$) lower *ghrl* expression compared to all the other experimental groups.

Finally, considering *lepa* gene expression (Figure 5c), CTRL, PG, F25, and F35 groups did not evidence significant differences among them, while showing a significantly ($p < 0.05$) higher expression compared to ROT and F32- groups.

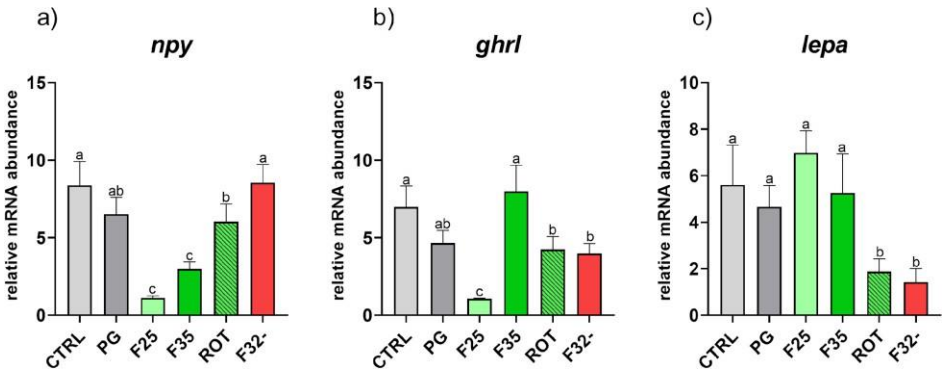

Figure 5. Relative mRNA abundance of genes involved in appetite regulation analysed in brain (*npy*), intestine (*ghrl*) and liver (*lepa*) samples from adult zebrafish. (a) *npy*, (b) *ghrl* e (c) *lepa*. Results are expressed as mean + SD (n = 5). [a-c] Different letters indicate statistically significant differences among the experimental groups.

Brain dopaminergic activity. As The CTRL and PG groups showed no significant differences among them in *drd2a* expression (Figure 6). Conversely, F25 and F35 groups showed significantly ($p < 0.05$) lower expression of *drd2a* compared to all other groups, excluding the ROT one.

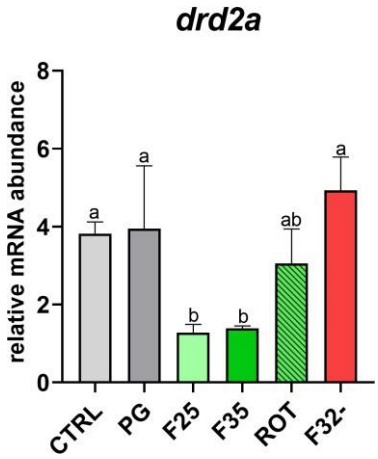

Figure 6. Relative mRNA abundance of *drd2a* involved in brain reward system analysed in brain samples from adult zebrafish. Results are expressed as mean + SD (n = 5). [a-b] Different letters denote statistically significant differences among the experimental groups.

Stress response. No significant differences were observed among experimental groups in the *nr3c1* gene expression (Figure 7).

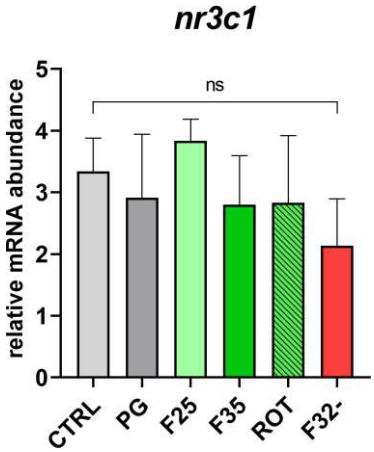

Figure 7. Relative mRNA abundance of *nr3c1* involved in stress response analysed in liver samples from adult zebrafish. Results are expressed as mean + SD (n = 5). ns, no significant differences.

Immunohistochemistry in the olfactory epithelium

The qualitatively analysis performed on the zebrafish olfactory epithelium (OE) revealed NPY-expressing olfactory neurons (OSNs) in all the different experimental groups. However, CTRL, PG, ROT, and F32- groups showed a homogeneous and diffused distribution of NPY-expressing neurons along the sensory epithelium of the lamellae (Figure 8d and Table 3). Conversely, both F25 and F35 groups showed a scarce and heterogeneous distribution of NPY-expressing neurons in the sensory area along the lamellae (Figure 8e and Table 3).

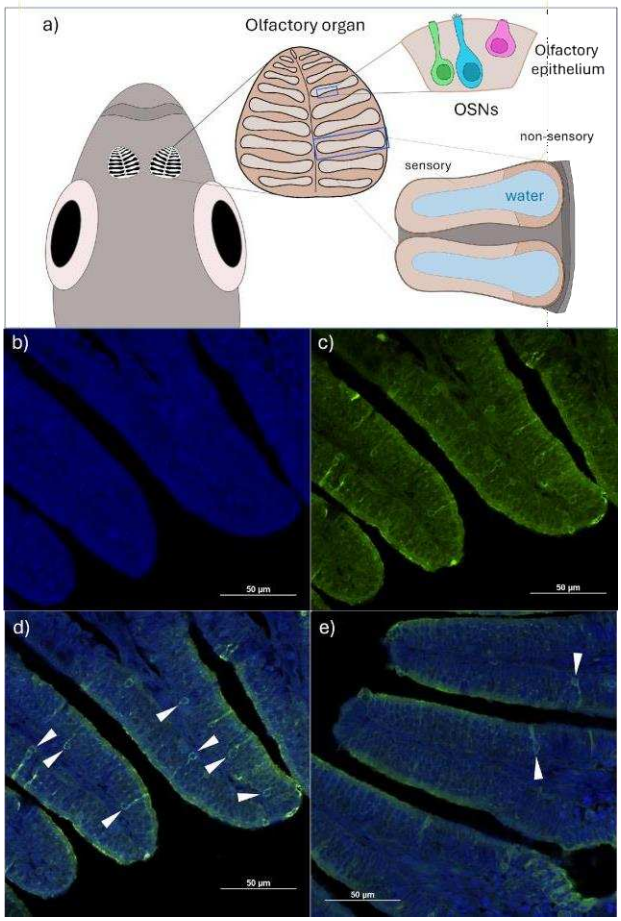

Figure 8. Neuropeptide Y (NPY) expression in the olfactory epithelium of zebrafish fed the different experimental diets. (a) Zebrafish olfactory organ, highlighting the morphology of epithelium arranged in lamellae. The central and medial region of each lamella includes a continuous sensory area, in which different olfactory sensory neurons (OSNs) are located, as well as a lateral non-sensory area (illustration by Conti F.); (b-c) DAPI (blue) correspond to nuclei while green (Alexa Fluor® 488) correspond to NPY (CTRL group); (d) representative transverse section of zebrafish olfactory epithelium from CTRL group showing NPY-expressing cells (arrowheads); (e) representative transverse section of zebrafish olfactory epithelium from F35 (caramel) group showing NPY-expressing cells (arrowheads). Scale bar = 50 µm.

Table 3. Qualitative analysis of the NPY-expressing neurons in the sensory area of the zebrafish lamellae, expressed as mean observation performed in all sections for each group.

	CTRL	**PG**	**F25**	**F35**	**ROT**	**F32-**
NPY-expressing cells distribution	+++	+++	+	+	+++	+++

Reproductive performances

Gonadosomatic index

Considering gonadosomatic index (Table 4), no significant differences were detected among the experimental groups ($p > 0.05$).

Table 4. Gonadosomatic Indexes of adult female zebrafish fed the experimental diets (GSI).

	CTRL	**PG**	**F25**	**F35**	**ROT**	**F32-**
GSI	8.69 ± 2.42	7.86 ± 0.67	7.16 ± 0.88	8.38 ± 1.38	10.16 ± 3.35	12.36 ± 5.02

Histological analysis of ovaries

Histology performed on ovary sections of all experimental groups revealed the presence of different oocyte classes (Figure 9). In particular, for the oocyte count, according to Randazzo et al. [54], oocytes were assigned to a pre- (PreV; stages I and II) or post-vitellogenic (PostV; stage III onward) stage, based on their morphological features: previtellogenic oocytes, with or without the cortical alveus presence (100–280 µm diameter); class III oocytes, characterized by enlarged yolk vesicles and vitellin membrane (280–740 µm diameter); and class IV oocytes, characterized by nuclear envelope break down and opaque ooplasm (>740 µm diameter). Since the class V oocytes ovulation occurs in few hours, they were rarely found in the analyzed zebrafish ovaries [40,41].

As reported in Table 5, both F25 and F35 groups showed a significantly ($p < 0.05$) lower percentage of pre-vitellogenic oocytes (72.19 ± 2.90 and 75.37 ± 2.69 %, respectively) and a significantly ($p < 0.05$) higher percentage of post-vitellogenic oocytes (27.08 ± 2.89 and 24.63 ± 2.69 %, respectively) compared to all other groups, which instead showed no significant differences among them ($p > 0.05$).

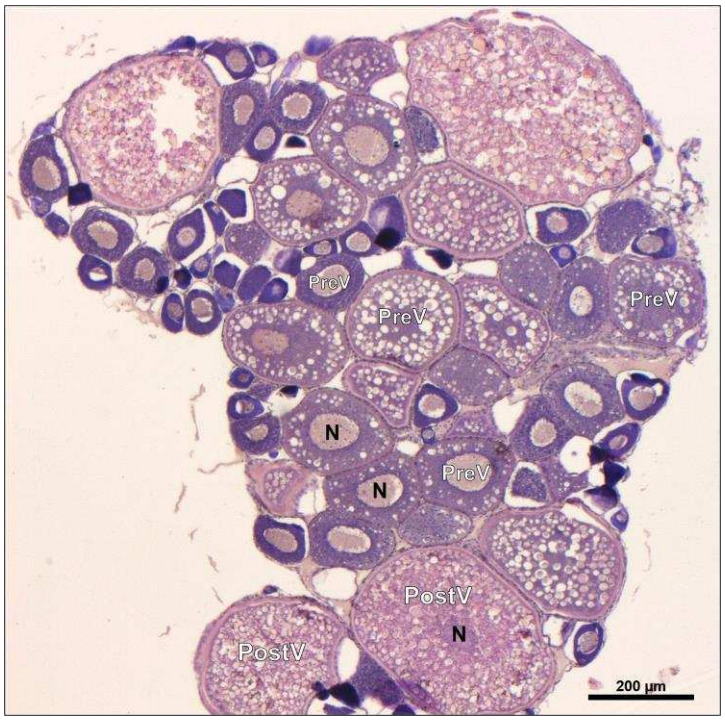

Figure 9. Oocyte developmental stages in zebrafish ovary. PreV, pre-vitellogenic stage; PostV, post-vitellogenic stage; N, nucleus. Scale bar: 200 µm.

Table 5. Percentage of pre-vitellogenic and post-vitellogenic oocytes.

	CTRL	PG	F25	F35	ROT	F32-
PreV	87.73 ± 3.02 [a]	85.92 ± 3.66 [a]	72.19 ± 2.90 [b]	75.37 ± 2.69 [b]	86.5 ± 1.50 [a]	87.09 ± 3.95 [a]
PostV	12.27 ± 3.02 [b]	14.09 ± 3.66 [b]	27.08 ± 2.89 [a]	24.63 ± 2.69 [a]	12.59 ± 1.13 [b]	12.31 ± 3.36 [b]

Results are expressed in percentage ([number of oocytes in each stage/total oocytes] × 100) as mean ± SD. [a-b] Different letters denote statistically significant differences among the groups ($p < 0.05$). PreV, pre-vitellogenic stage; PostV, post-vitellogenic stage.

Spawned eggs and hatching rate

As shown in Figure 10a, F25 and F35 groups were characterized by a significantly higher number of daily spawned eggs compared to the CTRL, PG, ROT, and F32- groups, within the 10-days period (485 ± 50, 485 ± 77, 576 ± 73, 579 ± 58, 388 ± 32, and 450 ± 82, for CTRL, PG, F25, F35, ROT and F32-, respectively). Additionally, ROT group were characterized by a significant lower number of daily spawned eggs compared to the CTRL and PG groups.

As regards the hatching rate (Figure 10b), no significant differences were observed among the experimental groups (84.1 ± 6.1, 87.9 ± 5.5, 84.5 ± 9.3, 86.3 ± 14.5, 79.8 ± 12.2, 80.7 ± 11.3%, for CTRL, PG, F25, F35 ROT and F32-, respectively).

Finally, no significant differences were detected in the percentage of unfertilized eggs, which was approximately 15% for all groups, in accordance with previous studies [64].

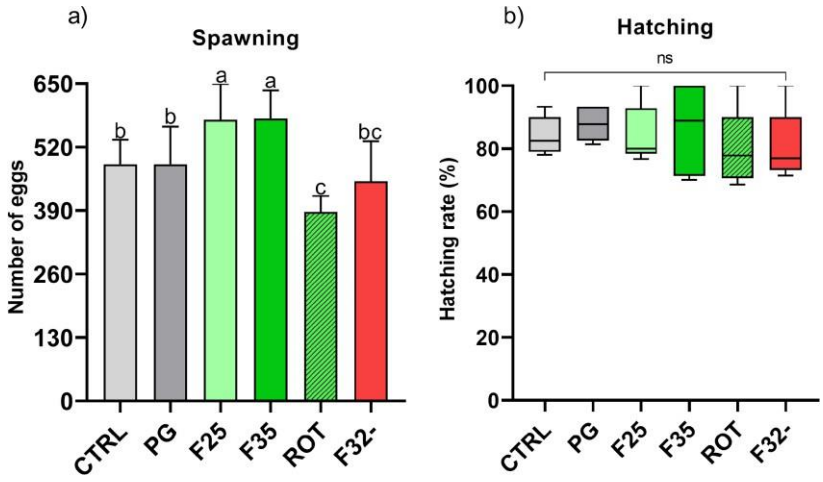

Figure 10. Total number of spawned eggs per day, and percentage of hatching rate observed in fish fed the different experimental diets. (a) Average daily number of eggs laid within a 10-day period. (b) Hatching rate expressed as percentage. Boxplots show minimum and maximum (whiskers), first quartile, median and third quartile (boxes). [a-c] Different letters indicate statistically significant differences between experimental groups ($p < 0.05$). Values are presented as mean ± SD (n = 10 spawning days, n = 9 for hatching rate).

Vitellogenin gene expression

Considering *vtg1* (Figure 11a), no significant differences were evident between CTRL and PG groups, while F25 was characterized by a significantly ($p < 0.05$) higher *vtg1* expression compared to all the other groups, except for F35.

Considering *vtg2* (Figure 11b), no significant differences were detected among CTRL and PG groups. F25 and F35 groups showed a significantly ($p < 0.05$) higher *vtg2* expression compared to both CTRL and F32- groups.

Finally, considering *vtg3* (Figure 11c), no significant differences were detected between CTRL and PG groups, while F35 group showed a significantly ($p < 0.05$) higher *vtg3* expression compared to all the other groups, that did not show significant differences among them.

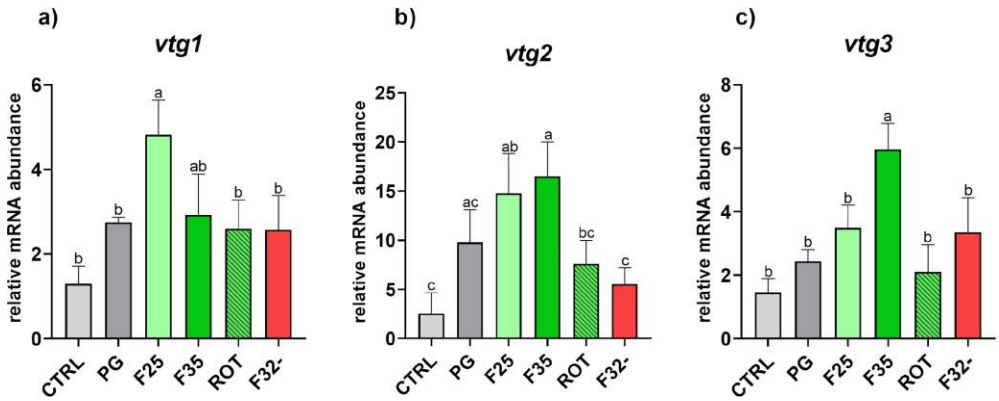

Figure 11. Relative mRNA abundance of genes involved in vitellogenin production analysed in liver from adult female zebrafish. (a) *vtg1*, (b) *vtg2*, and (c) *vtg3*. Results are expressed as mean + SD (n = 5). [a-b] Different letters indicate statistically significant differences among the experimental groups.

Discussion

Improving feeding techniques is a key aspect in fish farming to ensure better production yields that eventually have positive implications for both the sector's economy and the environment.

Considering this, the present study aimed to assess the role of synthetic flavors as potential and more sustainable feed attractants in a 6-month feeding trial on zebrafish, focusing on the female adult stage physiological responses. In fact, the zebrafish model has been considered suitable for studies for finfish aquaculture research, allowing to address all aspects of the life cycle, including nutrition, reproduction and welfare [35].

According to Conti et al. [33], both the solvent (PG) and all the tested synthetic flavors had no adverse effects on the overall fish health and on the target organs usually investigated when novel ingredients/additives are included in fish diets. In fact, no alterations in the intestinal architecture or differences in the histopathological indexes examined were detected; a physiological structure of the hepatic parenchyma was observed in all the experimental groups and no significant differences in the expression of the stress-related marker (*nr3c1*) was evidenced.

A part suggesting that the application of the present synthetic flavors did not affect fish welfare, the flavors attractive effect was confirmed during the feed intake experiment. Particularly, the maximum feed intake achieved was observed in fish fed diets implemented with cheese (F25) and caramel (F35) flavors. Maximizing feed consumption in aquaculture is a remarkable achievement, as rapid and complete feed intake allows faster fish growth, thus shortening production time while decreasing feed losses [15,53]. These results were further supported by the higher final body weight of the fish and the consequent upregulation of the growth-promoting factor *igf1*, as already demonstrated by Conti et al. [33] during zebrafish early development.

It is well established that fish feeding behavior is driven by appetite regulatory mechanisms, as a consequence of the interaction between signals (both orexigenic and anorexigenic), produced at the gastrointestinal tract and brain level [18,20]. In addition, foraging is influenced by a wide range of water-soluble compounds which are discriminated by the fish olfactory system [65]. Neurohormonal signals can influence this peripheral circuit, including the action of the olfactory sensory neurons (OSNs), *via* a nutritional state-dependent modulation [61]. For example, a reduction in Neuropeptide Y signaling in fish olfactory system has been associated to a higher nutrient availability [23,24], while an increase in the number of NPY-expressing OSNs was associated to starvation [61]. On this basis, the lower brain *npy* gene expression level coupled with the lower NPY immunoreactivity detected in the zebrafish olfactory epithelium sustained the higher satiety state of groups fed both cheese (F25) and caramel (F35) flavored diets.

Nutrient availability, as well as malnutrition and starvation, are known to influence one of the most powerful orexigenic signals synthetized at peripheral level, the ghrelin [19]. Increasing levels of this hormone, synthetized in the intestine, are known to stimulate *npy* expression at central level [20]. Thus, a higher feed intake can decrease peripheral *ghrl* expression, which in turn lowers *npy* expression at the central level. This *scenario* was evident only in group fed cheese-flavored diet, confirming a more satiated state in this group. Considering the group fed caramel-flavored diet, *npy* expression at central level supported the above-

mentioned outcomes, while a non-obvious result was obtained for *ghrl* gene expression. This latter result can be related to the fact that other physiological mechanisms, such as the gastrointestinal tract motility, digestive, and absorption functions, can have a deep impact on *ghrl* gene expression in the intestine [66–68].

As regards leptin, its gene expression in groups CTRL, PG, F25 (cheese) and F35 (caramel), suggested an adequate nutritional status of the fish since the levels of this hormone are directly related to the body energy reserves and thus feed intake [21,22].

Appetite in teleost is also strictly related to the monoaminergic system, that influences feeding behavior along with the above-mentioned homeostatic neuroendocrine pathways [25]. A highly palatable feed can promote its ingestion because of its hedonic properties, actively involving the dopamine receptors in fish brain reward system [26]. Among the different dopamine receptors, Drd2 is suggested to be strongly related to feed intake in vertebrates [69,70]. Additionally, during a time-prolonged positive stimulation, dopamine receptors downregulation has been associated to the reinforcement mechanisms [71]. Accordingly, in the present study, which lasted for six months, *drd2a* was downregulated in caramel and cheese groups, supporting the attractive effectiveness of F25 and F35 flavors as positive stimuli.

Feed intake is also closely related to the reproductive functions in teleost. In fact, it is well established that malnourishment condition or scarce feed/nutrient availability can have negative implications on fish reproduction [47]. The quality of maternal nutrition is crucial especially during oocyte development, a process that requires proper allocation of macromolecules such as vitellogenin [46,72]. In this regard, oocyte development is well known in zebrafish. In stage I, oocytes begin to enlarge, and follicles start to form. Subsequently, cortical alveoli accumulate within the oocytes (Stage II), while stage III oocytes undergo vitellogenesis, resulting in significant dimensional increase. In stage IV oocyte, yolk vesicles begin to fuse with each other, resulting in occyte maturation. Finally, when mature eggs are ovulated and ready for spawning is referred as stage V [40,41].

Histological analysis on the ovaries from cheese (F25) and caramel (F35) groups, evidenced a high number of post-vitellogenic oocytes, reflecting an increased maturation state. In addition, since the vitellogenin uptake in oocytes establishes their transition from previtellogenic to mature stages [40,41], this latter result was further supported by *vtg* gene expression. In fact, the detected upregulation of all VTGs isoforms analyzed, in specimens fed cheese and caramel diets, suggested the promotion of oocytes maturation process in these experimental groups, which was further supported by a higher spawning rate. Having ingested more feed, specimens from these groups were likely more capable to invest in high-metabolic demanding physiological mechanisms such as reproduction [43]. This result may also be explained by the fact that such specimens produced more eggs as a consequence of their bigger size [73], reflecting that a higher feed intake supported the growth and reproductive capabilities of the specimens. Nevertheless, despite the differences in the average daily number of spawned eggs among groups, a comparable hatching rate was observed, a result easily explainable by the fact that the administered dietary formula was the same in all experimental groups [47]. Finally, in accordance with previous outcomes [33], and considering the overall results obtained in the present study, the ROT (rotation) group was not a reliable feeding scheme. The repeated positive flavor/stimulus shift

(cheese/caramel) did not provide the expected positive results, since ROT group was designed to avoid the possible adaptation of fish olfactory receptors, in response to a repeated stimulation [49]. Concurrently and in accord to a previous study [33], the repulsive effect of the coconut flavor (F32-) was reduced by the interaction with the feed odor, or by a possible adaptation of the fish to the flavor.

Conclusions

Both CTRL and PG diets showed comparable results, while the attractive synthetic flavors tested confirmed their attractive role also in fish exposed over a long-term period. The provision of both diets containing cheese- or caramel-attractive flavors resulted in increased feed ingestion via appetite- and olfactory stimuli, all mediated by the influence of the feed hedonic properties and a higher growth rate. Moreover, the inclusion of attractive flavors positively promoted the fish reproductive performances, being the fish able to invest to a greater extent in this high metabolic demanding process. Finally, in accordance with a previous study [33], the ROT group was not a reliable feeding scheme while the repulsive coconut flavor led to controversial results. In conclusion, the present study demonstrated the promising role of synthetic flavors in advancing fish feeding strategies. Using this new generation additives will reduce the natural dependence on the conventional feeding stimulants, and relies on a standardized manufacturing process, ensuring consistent attractive effectiveness within aquafeeds.

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Chapter 4

Synthetic feed attractants in European seabass (*Dicentrarchus labrax*) culture: effects on growth, welfare, and appetite stimulation

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Abstract

Improving feed palatability is one of the ongoing challenges of aquaculture sector in light to reduce the quantity of unconsumed feed and to avoid worsening of water quality. The introduction of standardized and cost-effective processes to produce synthetic flavors as feed attractants can represent a sustainable solution to solve issues related to feed waste and to aquaculture impact on the environment. In this context, the present study aimed to investigate the potential role of synthetic flavors as feed attractants in European seabass (*Dicentrarchus labrax*), a carnivorous and commercially relevant species for Mediterranean aquaculture. Two attractive (F25, cheese; F35, caramel) and one repulsive (F32-, coconut) flavors were separately added (1% w/w) to a commercial diet for European seabass and then tested, during a 90-day feeding trial, to evaluate their effects on fish welfare, zootechnical performance, and modulation of monoaminergic systems. All the tested synthetic flavors did not affect the fish overall health status. The F35 flavor was able to enhance fish feed intake and growth as well as positively modulate the monoaminergic activity, proposing itself as a more suitable attractive solution compared to the F25 one. The weekly rotation scheme between F35 and F25 during the whole trial (ROT group) led to suboptimal results compared to the administration of F35 alone. The F32- flavor was not possibly perceived as a strongly negative stimulus in this fish species, not affecting fish zootechnical performance. The present study demonstrated the effectiveness of attractive synthetic flavors in increasing diet palatability also in a carnivorous species of commercial interest, with promising implications on sustainability and cost-effectiveness of feeding practices in aquaculture.

Introduction

In a blue economy context, the aquafeed industry is achieving considerable progress in enhancing resource utilization [1,2]. In fact, the use of fish meal and oil for diet formulations, despite being ideal ingredients with a well-balanced amino acid profile, are no longer economically and environmentally sustainable due to their increasing cost and the volume of wild-caught forage fish needed [3–5]. Therefore, the aquafeed production sector was driven to explore several alternative ingredients or feed additives [6–9]. Particularly, the plant-derived raw materials still remain the major alternative to fish meal and oil even though they affect the food-feed competition and may potentially have negative effects on gut health, especially in carnivorous fish [10–12]. Furthermore, the inclusion of high percentage of ingredients of vegetable origin can also reduce the aquafeeds palatability [13]. In fact, the off-flavor substances present in these ingredients, like alkaloids, can affect the attractiveness of the feed, resulting in reduced feed intake and thus compromising growth performances of farmed fish [14,15]. The consequently higher amounts of unconsumed feed can have long-term effects on both the farm economics, especially considering that aquafeeds are the most expensive component in aquaculture, and on the aquatic environment, deteriorating water parameters and leading to eutrophication [16,17]. Enhancing feed palatability, particularly for carnivorous fish fed diets with reduced levels of marine-derived ingredients, is a crucial issue to be considered [18]. In this regard, feed attractants can be incorporated in aquafeeds formulations to positively modify their flavor and thus to enhance their consumption by the fish [19]. Organoleptic properties of highly palatable diets are in fact able to drive the taste preferences of fish, acting on their chemosensory capabilities [20]. Molecules released by the feed attractants are received by the fish olfactory and gustatory systems, eventually activating the central nervous system and inducing reactions such as feed-seeking, ingestion, and continuation of feeding [21]. In addition, the stimulation of these systems in the buccal region can trigger gastric secretion in anticipation of food intake, promoting better digestion and absorption through the production of essential peptides and hormones that regulate digestive functions, gut motility, feed intake, and metabolism [22]. In fact, the brain is the key site that receives and integrates information about the nutritional state and energy level of the organism [23]. In fish, monoaminergic systems are part of the neural network that controls feeding, and both serotonin- (or 5-hydroxytryptamine) and dopamine-receptors are directly involved [24]. In this regard, the hedonic properties of feed, resulting from its palatability, are able to influence these mechanisms such as the activation of the fish brain reward system, stimulating feed intake [25,26].

Numerous studies have demonstrated that some specific compounds like L-amino acids, small chain peptides, betaine, and nucleotides are potent attractants for fish [27,28]. Particularly, it has been shown that the nucleotides found in the muscle extract of jack mackerel produced the strongest feeding stimulatory response in greater amberjack (*Seriola dumerili*) [29]. In fact, these substances are abundantly found in marine by-products such as meals or extracts derived from forage fish, mollusks, squids, and shrimps, leading to the inclusion of these raw materials in aquafeeds as natural attractants [30,31]. However, the recurring dependence on marine resources still rises unsustainability concerns [32], besides considering also that their attractive

effect is highly variable depending on their origin, quality, freshness, and manufacturing practices [3,33]. The ongoing challenge on finding effective feed attractants for carnivorous fish, without compromising the sustainability of the aquafeed sector, has led to the dietary inclusion of the above-mentioned molecules, exploiting their potential attractive role without relying on marine resources. In this context, Yesilayer and Kaymak [18] found that 1% of crystalline betaine supplementation in a diet rich in fish meal and oil (with 25% of soybean meal) positively influenced growth performance and feed utilization in rainbow trout (*Oncorhynchus mykiss*) juveniles. Differently, amino acids, despite being recognized as the major class of compounds with attractive effect, present several limitations due to their concentration and interaction with other dietary components as well as to their different effectiveness in relation different fish species and life cycle stages [34,35]. Hence, when these molecules are singly provided, they are generally considered less effective in attracting fish compared to the extracts of marine-derived raw materials [28,34].

Synthetic flavours derived from standardized processes have been recently proposed as novel and promising alternative to the traditional feed attractants [36]. Particularly, a preliminary study conducted during zebrafish (*Danio rerio*) early development demonstrated that the dietary supplementation with 1% (w/w) of attractive synthetic flavours had positive effects on appetite stimulus, feed ingestion, and, consequently, on the growth of fish [36]. Zebrafish has been recognised as an optimal model for fish nutrition studies applicable in the aquaculture sector [37], and preliminary results obtained in this species can be a crucial starting point for further investigations in farmed fish.

In this context, the aim of the present study consists in testing the effectiveness same synthetic flavours previously tested in zebrafish [36] on European seabass (*Dicentrarchus labrax*), during a 90-day feeding trial, evaluating the fish physiological responses. Through a multidisciplinary laboratory approach including biometric measurements, histological and molecular investigations, known to be necessary when new aquafeed ingredients or additives are tested, emphasis was given to fish zootechnical performances and welfare as well as the brain monoaminergic systems modulation. The European seabass is a strictly carnivorous species with a high market value and of particular importance in the aquaculture sector of the Mediterranean region [38]. The production rates of this species are increasing in response to the ever-growing demand of seafood and feed costs constitute the wider proportion of the expenses of such production [39]. The introduction of synthetic flavours as feed attractants can allow to reduce feed waste and to ensure growth performance and productivity, by increasing the diet palatability. In addition, the shift towards more sustainable and innovative solutions in aquafeed formulation is a positive step towards promoting eco-friendly practices in the aquaculture sector.

Materials and Methods

Ethics

The feeding trial and all the experimental procedures involving animals were conducted according to EU legal frameworks relating to the protection of animals used for scientific purposes (Directive 2010/63/EU). The study was approved by the Ethics Committee of the Marche Polytechnic University (Ancona, Italy) and the Italian Ministry of Health (Aut N° 453/2023-PR). Fish suffering was minimized by using the MS222 anesthetic (1 g/L; Merck KGaA, Darmstad, Germany).

Synthetic flavors and production of experimental diets

The two attractive (F25, cheese; F35, caramel) and one repulsive (F32-, coconut) flavours were produced by To Be Pharma S.r.l. (S. Egidio alla Vibrata, Teramo, Italy) following the same composition reported in Conti et al. [36], in compliance with the current sector-specific legislation, specifically Regulation (EC) No 1334/2008 and 1333/2008. Briefly, each synthetic flavor consisted of a liquid blend of mixed aromatic chemicals added to 1,2-propanediol (propylene glycol; PG) as a functional solvent (all the substances used are recognized as safe for use by the Flavor and Extracts Manufacturers Association and the Food and Drug Administration -Agency for Toxic Substances and Disease Registry 2007). The safety of the flavors for both humans and animals has been confirmed through the technical data sheet and the Material Safety Data Sheet (MSDS), ensuring the respect of the regulatory standards and the safety for consumption.

A commercial diet for European seabass (Ecoprime, Veronesi, VR, Italy), rich in land animal proteins (LAPs), was used as control diet (CTRL). For the available details of the control diet please see https://fishfeed.eu/IT/alimentazione-pesce/mangime-Spigola/9/8/2/Spigola-ECOPRIME-3.aspx. Then, four experimental diets were prepared starting from the commercial CTRL diet by adding 1% (w/w) of PG, F25 flavor (attractive), F35 flavor (attractive), or F32- flavor (repulsive) (named PG, F25, F35, or F32- diets, respectively). The PG and all the flavours were daily added at 1% (w/w) to the daily feed ration using a micropipette. Subsequently, the mixture was vigorously mixed to ensure a uniform flavours distribution in the feed. This process was implemented to guarantee the consistency in the experimental conditions.

Experimental design and zootechnical parameters

Five hundred and forty European seabass juveniles (initial body weight: 72.48 ± 8.04 g) provided by Panittica Pugliese (Torre Canne di Fasano, Brindisi, Italy) were acclimated for two weeks in a single 1000 L tank with mechanical and biological filters and a UV lamp (Panaque s.r.l., Viterbo, Italy) that ensured the following water parameters: temperature, 21.0 ± 0.5 °C; salinity 30 ± 0.5 ‰; ammonia and nitrite < 0.05 mg/L; nitrate < 10 mg/L. At the end of acclimation period, fish were randomly divided into six experimental groups (in triplicate) as follows: (i) CTRL group, fish fed the CTRL diet; (ii) PG group, fish fed PG diet; (iii) F25 group, fish fed F25 diet; (iv) F35 group, fish fed F35 diet; (v) F32- group, fish fed F32- diet; (vi) ROT group, fish fed the two attractive diets (F25 and F35), each administered singularly in a weekly rotation scheme, in light to avoid a possible olfactory receptors adaptation in response to a long-term stimulation [40]. Each experimental

groups (90 fish per group; 30 fish per tank) consisted of three 500L tanks provided with mechanical and biological filtration (Panaque).

The duration of the feeding trial was 90 days during which the feed consumption was daily monitored, providing a pre-weighed quantity of feed corresponding to 1.5% of fish body weight (in agreement with the feed company instructions in relation to fish size and water temperature). The quantity of feed was adjusted every 14 days by weighing all the specimens from each tank. Seabass were hand-fed the experimental diets and the eventually uneaten feed was daily recovered 15 min post-administration by siphoning, dried overnight in an oven at 40 °C, and weighted. The daily feed intake data were calculated by subtracting uneaten feed from the feed administered and then expressed as an average percentage of daily feed ingested. The 15-minute duration was chosen based on the maximum duration of the behavioural preference test for flavours selection, as described in the study by Conti et al. [36], along with the fact that an effective feed attractant has to stimulate a rapid ingestion of the feed within the first minutes of administration [41].

During the whole trial, a visual inspection of tanks was daily carried out to check the eventual presence of dead specimens.

At the end of the feeding trial, after 24 hours fasting, all the fish were euthanized with a lethal dose of MS222 (1 g/L, Merck KGaA), and then individually weighted. Data were used to calculate: (i) weight gain: WG (g) = final body weight – initial body weight; (ii) feed conversion ratio, FCR = total weight of feed consumed / (final body weight – initial body weight); (iii) relative growth rate: RGR (%) = [(final body weight-initial body weight) /initial body weight] × 100; (iv) specific growth rate: SGR (%) = [(ln final body weight – ln initial body weight) / time (days)] × 100.

Survival rate was calculated as follows: SR (%) = (final number of fish / initial number of fish) × 100.

Finally, samples from brain, liver, and distal intestine, were collected and properly stored for further analyses, as reported in the below-described sections.

Histological analysis

Samples of liver and distal intestine were collected from 10 randomly selected fish per tank (n = 30) and fixed for 24 h at 4°C by immersion in Bouin's solution (Merck KGaA). Samples, after being dehydrated in ethanol solutions (80, 95, 100%), washed with xylene (Bio-Optica, Milan, Italy), and included in paraffin (Bio Optica), were cut with a microtome (Leica RM2125 RTS, Nussloch, Germany) to obtain section of 5 μm in thickness. Sections were stained with Mayer hematoxylin and eosin Y (EE; Merck KGaA) or Periodic Acid Schiff (PAS; Bio-Optica, Milan, Italy) and were then observed using a Zeiss Axio Imager.A2 microscope (Zeiss, Oberkochen, Germany). Images were acquired using a combined colour digital camera (Axiocam 105, Zeiss) and measurements were taken with the ZEN 2.3 software (Zeiss). For each sample, the morphometric and histopathological evaluations were performed on three transversal sections collected at intervals of 200 μm. The histopathological parameters measured for both distal intestine and liver sections were considered as previously described in Zarantoniello et al. [42]. Specifically, for distal intestine, the mucosal fold height (all

the undamaged and non-oblique folds were considered) and submucosa width were measured. In addition, the events of mucosal folds fusion, the incidence of inflammatory influx in the submucosa, and the presence of supranuclear vacuoles were considered during the inspection of each section. As regards liver, the EE staining was used to assess hepatocytes morphology, the presence of pathological alterations, and the degree of fat accumulation, while the PAS staining allowed to highlight glycogen deposition in hepatocytes. The fat fraction percentage was quantified on areas with no blood vessels or bile ducts using ImageJ software (setting a homogeneous threshold value) and expressed as percentage of the area occupied by fat on the total hepatic parenchyma.

Molecular analyses

Total RNA was extracted from samples of brain and intestine of 5 fish per tank (15 per experimental group) using TRIzol™ reagent (Invitrogen, Waltham, MA, USA) according to Cionna et al. [43]. Then, after checking the RNA final concentration (Nanophotometer P-Class, Implen, München, Germany) and the integrity, the complementary DNA was synthesized from 1 µg of total RNA using the iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA, USA).

The relative mRNA abundance of genes involved fish brain reward system (dopamine receptor D3, *drd3*), serotonergic system (5-hydroxytryptamine receptor 1A, *5ht1a*), immune response (interleukin 1β, *il1b*; interleukin 10, *il10*; tumor necrosis factor alpha, *tnfa*) was performed on brain and intestine samples, respectively. As reference genes, beta-actin (*β-actin*) and 18S ribosomal protein (*18s*) were used. The primers sequences used in the present study are reported in Table 1. The real-time quantitative PCR were performed in an iQ5 iCycler thermal cycler (Bio-Rad, Hercules, CA, USA) following the protocol described in Randazzo et al. [44]. At the end of each cycle, the fluorescent was monitored, and the melting curve presented a single peak for every product. Two no template controls were added for each reaction to guarantee absence of contamination. Amplification products were sequenced, and homology was verified. The qPCR data were processed using the iQ5 optical system software version 2.0 (Bio-Rad), including GeneEx Macro iQ5 Conversion and genex Macro iQ5 files.

Table 1. Sequences, annealing temperature (A.T.), and NCBI IDs of the primers used in the present study.

Gene	Forward Primer (5'–3')	Reverse Primer (5'–3')	A.T. (°C)	NCBI ID
5ht1a	GGGTTGTTTTCAGGACCAAG	CGCAGAAAGGAGAAGAGCAA	58	XM_051426243
drd3	GAATGAGCTGCGAGGTGAA	CGTGGGTTGTTGGTATGAGA	58	XM_051411236
il1b	AACTCCAACAGCGCAGTACA	AGACTGGCTTTGTCCACCAC	58	AJ_311925
il10	GCAGTCCCATGTGCAACAAC	TGCTACTGAACCTACGTCGC	59	AM_268529
tnfa	GACTGGCGAACAACCAGATT	GTCCGCTTCTGTAGCTGTCC	59	DQ_070246
b-actin (hk)	GGTACCCATCTCCTGCTCCAA	GACGTCGCACTTCATGATGCT	60	AJ_537421
18s (hk)	AGGGTGTTGGCAGACGTTAC	CTTCTGCCTGTTGAGGAACC	60	XM_051390998

Statistical analyses

The tanks were used as experimental unit for data related to survival rate and zootechnical performance, while fish were considered as experimental unit for all the remaining analyses. After being checked for normality (Shapiro–Wilk test) and homoscedasticity (Levene's test), data from each analysis were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc test (software package Prism 8, Graphpad Software version 8.0.2, San Diego, CA, USA). Significance was set at $p < 0.05$.

Results

Zootechnical performance

Table 2 reports survival rate, growth performance, and daily feed intake of European seabass fed the experimental diets. The survival rate was 100% in all the experimental groups. Considering growth performance, fish fed F35 were characterized by significantly ($p < 0.05$) higher values of final body weight, weight gain, and relative and specific growth rates compared to CTRL, PG, F25, and F32- groups that did not show significant differences among them. In the ROT group, all these indexes were lower compared to those observed in the F35 group, without statistically significant differences. In addition, considering feed conversion ratio, no significant differences were detected among experimental groups ($p > 0.05$). Finally, as regards the daily feed intake, fish fed F35 and ROT diets showed a significantly ($p < 0.05$) higher percentage of feed ingested respect to all the other groups (with the exception of F25) which did not show significant differences among them.

Table 2. Survival rate and zootechnical performance of European seabass fed the experimental diets.

	CTRL	**PG**	**F25**	**F35**	**ROT**	**F32-**	***p-Value***
SR (%)	100	100	100	100	100	100	-
IBW (g/fish)	67.8 ± 6.3	71.5 ± 8.9	72.6 ± 8.1	75.3 ± 7.8	71.6 ± 7.8	75.9 ± 8.3	0.8291
FBW (g/fish)	196.0 ± 35.4 ^{a}	180.1 ± 17.6 ^{a}	194.1 ± 15.1 ^{a}	256.1 ± 16.1 ^{b}	219.9 ± 19.7 ^{ab}	176.2 ± 10.8 ^{a}	0.0043
WG (g/fish)	120.9 ± 25.8 ^{a}	108.1 ± 17.6 ^{a}	122.2 ± 15.2 ^{a}	194.1 ± 26.1 ^{b}	167.9 ± 32.7 ^{ab}	110.4 ± 13.9 ^{a}	0.0026
RGR (%)	181.6 ± 22.7 ^{a}	160.1 ± 24.5 ^{a}	169.7 ± 21.0 ^{a}	251.9 ± 20.2 ^{b}	210.3 ± 27.2 ^{ab}	176.6 ± 19.3 ^{a}	0.0034
SGR (%)	1.7 ± 0.1 ^{ab}	1.5 ± 0.2 ^{ab}	1.6 ± 0.1 ^{ab}	2.2 ± 0.2 ^{c}	1.8 ± 0.2 ^{bc}	1.3 ± 0.1 ^{a}	0.0003
FCR	0.96 ± 0.08	1.17 ± 0.09	1.17 ± 0.09	0.96 ± 0.10	1.04 ± 0.09	1.20 ± 0.15	0.0359
Daily FI (%)	75.4 ± 2.7 ^{a}	74.1 ± 2.2 ^{a}	80.4 ± 3.2 ^{ab}	90.1 ± 5.6 ^{b}	87.6 ± 2.5 ^{b}	73.9 ± 6.1 ^{a}	0.0008

Values are shown as mean ± SD (n = 3). Abbreviations: SR, survival rate; IBW, initial weight; FBW, final weight; RGR, relative growth rate; SGR, specific growth rate; FCR, feed conversion ratio; FI, feed intake.

Histological analyses

Considering distal intestine (Figure 1), no morphological or pathological alterations were observed in all the experimental groups. Specifically, no significant differences were observed among the experimental groups in terms of both mucosal fold height and submucosa width (Table 3). Furthermore, distal intestine samples from all the experimental groups highlighted a physiological condition, showing a scarce lymphocytes infiltration, absence of supranuclear vacuoles, and episodes of mucosal folds fusion were isolated.

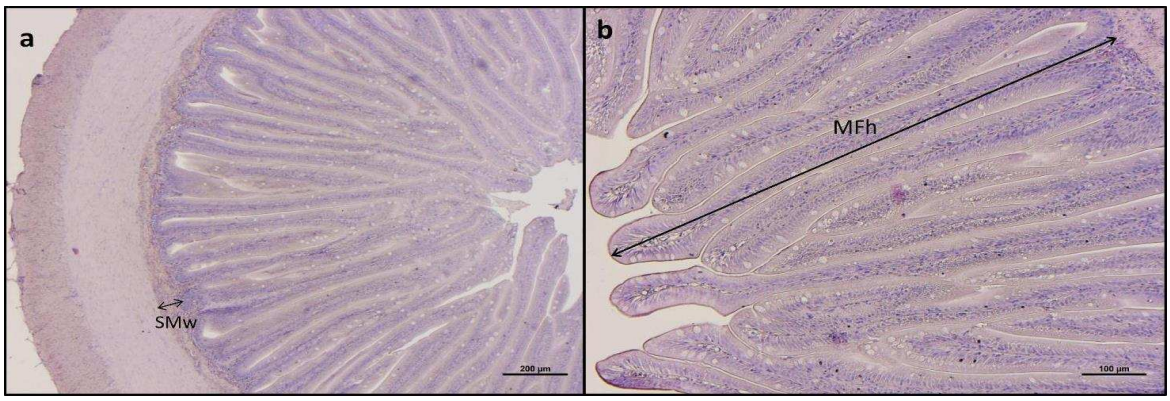

Figure 1. Histological evaluation of intestine. (**a**) Example of histomorphology of distal intestine from European seabass fed the F35 diet with measurement criteria for submucosa width (SMw); (**b**) example of histomorphology of distal intestine from European seabass fed the PG diet with measurement criteria for the mucosal fold height (MFh). Scale bars: (**a**) 200 µm; (**b**) 100 µm.

Table 3. Histological parameters considered in distal intestine and liver of European seabass fed experimental diets.

	CTRL	PG	F25	F35	ROT	F32-
Distal intestine						
Mucosal fold height	1023.0 ± 73.9	969.4 ± 52.0	977.7 ± 71.9	942.2 ± 75.2	1097.0 ± 40.2	918.0 ± 30.1
Submucosa width	38.98 ± 0.04	40.92 ± 2.50	45.03 ± 2.64	40.31 ± 2.05	42.34 ± 1.54	45.86 ± 2.04
Hepatic parenchyma						
Fat fraction (%)	48.9 ± 3.5 [a]	50.7 ± 4.5 [a]	49.7 ± 6.5 [a]	60.5 ± 3.9 [b]	58.5 ± 2.6 [b]	47.8 ± 7.3 [a]

Values are presented as mean ± SD (n = 30)

As regards liver, fish from all the experimental groups exhibited a physiological structure of hepatic parenchyma (Figure 2). However, liver sections revealed a variable degree of fat accumulation among the experimental groups. Specifically, F35 and ROT groups exhibited a significantly higher percentage of fat fraction compared to all the other experimental groups (Table 3), with hepatocytes characterized by an abundant intracytoplasmic accumulation of fat. Finally, no differences were evident among the experimental groups in terms of glycogen deposition, classified as modest in each PAS-stained section (Figure 2 c).

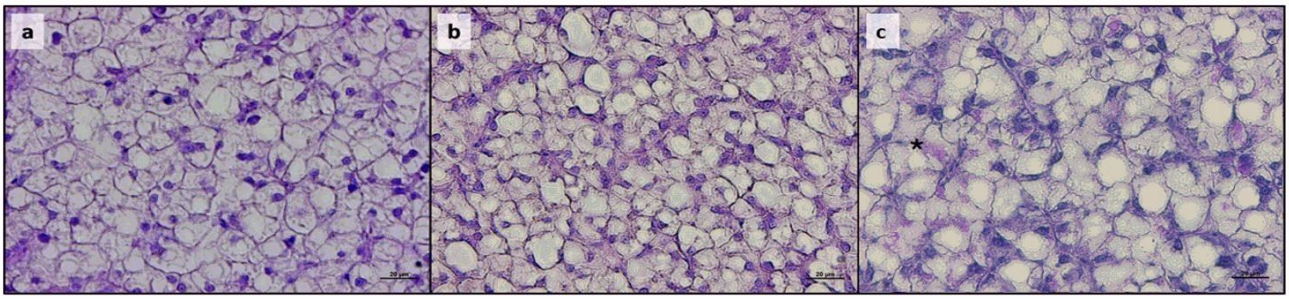

Figure 2. **Histological evaluation of liver.** Example of histomorphology of liver from European seabass fed the experimental diets. **(a)** CTRL; (**b**) F35; (**c**) ROT. Stainings: (**a**-**b**) EE; (**c**) PAS, asterisk indicate glycogen deposition. Scale bars: 20 µm.

Real-Time qPCR results

Monoaminergic system. Considering the *5ht1a* relative gene expression (Figure 3 a), F35 and ROT groups showed a significant ($p < 0.05$) upregulation compared to both CTRL and F32- groups, while F25 and F32- groups did not show significant differences compared to CTRL group. No significant differences were detected between CTRL and PG groups.

Considering *drd3* relative gene expression (Figure 3 b), F32- and ROT groups showed a significant ($p < 0.05$) upregulation compared to the other experimental groups, which did not show significant differences among them.

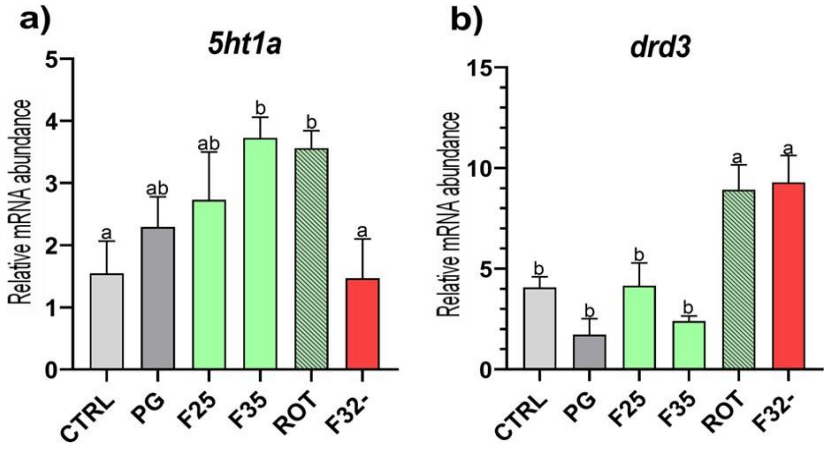

Figure 3. Relative mRNA abundance of markers related to monoaminergic systems. Real-time qPCR were performed on brain samples of European seabass fed the experimental diets. Results are expressed as mean ± SD (n = 5). [a–b] Different letters denote statistically significant differences among the experimental groups.

Immune response. Considering the expression of markers involved in immune response (Figure 4), groups fed the F35 and F32- diets showed a significant ($p < 0.05$) upregulation of *il1b*, *il10*, and *tnfa*, compared to the other groups which did not show significant differences among them.

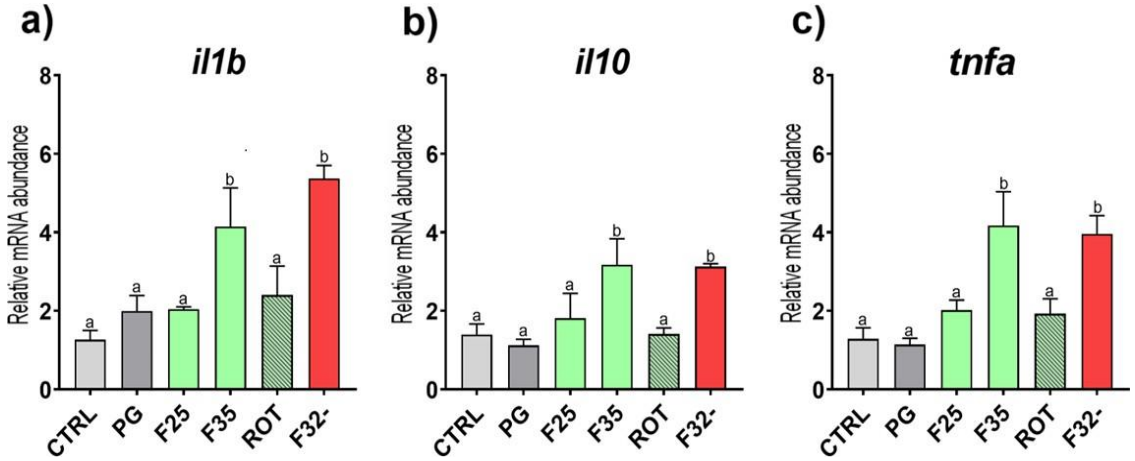

Figure 4. Relative mRNA abundance of genes involved in the immune response. Real-time qPCR were performed on distal intestine samples of European seabass fed the experimental diets. Results are expressed as mean ± SD (n = 5). [a–b] Different letters denote statistically significant differences among the experimental groups.

Discussion

Evaluating the effectiveness of synthetic flavours as feed attractants in diets intended for carnivorous fish species of commercial interest, due to some limitations of the currently used aquafeed formulations, is of great importance for improving feeding practices within the aquaculture sector. The synthetic flavours used in the present study were preliminary tested in a previous study conducted on zebrafish early development [36], demonstrating their safety for fish and, regarding the attractive ones, their effectiveness in enhancing appetite stimulus, feed ingestion, and growth. Considering that zebrafish is a proper model organism for nutrition studies [37] and, differently from most of the farmed fish species in the Mediterranean region, is omnivore [45], the present study aimed to investigate the suitability of the same synthetic flavours on a carnivorous species of commercial interest, the European seabass.

As a first consideration, the overall health status of target organs directly involved in feed processing must be explored when novel additives are included in the feed formulation. In the present study, the PG and all the tested synthetic flavors had no adverse effects on liver and distal intestine, as widely demonstrated by the histological analysis. However, it should be pointed out that, fish fed caramel and coconut diets were characterized by an upregulation of immune-related markers (*il10*, *il1b*, *tnfa*) in distal intestine, despite the clear absence of inflammatory process evidenced by the histological analyses. Pro-inflammatory cytokines are crucial in triggering the inflammatory response, acting on the proliferation of T and B lymphocytes as well as macrophages, thereby promoting the migration of immune cells to the site of inflammation [46,47]. Fish interleukins can be also involved in the regulation of different physiological processes besides immune function, such as muscle growth and metabolism [48], adding another layer of complexity to the observed results. These various roles of interleukins could contribute to the discrepancies in the study outcomes, emphasizing the need for further research to elucidate the non-obvious relation between the provision of synthetic flavours and fish cytokines relative gene expression. The lack of adverse effects associated with the base solvent used was clearly demonstrated by the comparable results obtained between fish fed CTRL and PG diet.

Together with the maintenance of an optimal welfare status of fish, the main goal of the dietary addition of feed attractants is ensuring palatability in order to support high production rates. In the present study, fish fed a commercial diet supplemented with the caramel synthetic flavor alone or in a rotation scheme with the cheese one (F35 and ROT groups, respectively) showed a significantly higher daily feed intake compared those fed CTRL, PG, and coconut diets, confirming, even in this species, the attractive effectiveness of the caramel flavor, as already previously demonstrated in zebrafish [36]. The higher daily feed intake in these groups, over the whole feeding trial, led to direct consequences on the amount of energy available for fish to support growth performance and storage of nutrients. In fact, both the caramel and ROT groups showed the highest weight gain and growth rates (even if results from ROT group were not statistically different from those obtained by the other groups) and exhibited a significantly higher fat percentage in the liver, which is one of the main sites

of lipid storage in several marine fish species, including European seabass. This finding is consistent with a previous study on rainbow trout [49] that demonstrated how the incorporation of feed attractants in a low palatable diet (based on potato protein concentrate) was able to enhance fish feed intake, ensuring an optimal sustain to growth. In the present study, no significant differences were observed in FCR, since the administered diet was the same in all experimental groups. Therefore, the increased growth detected in groups fed on diets added with the attractive flavours can be only imputable to the increased feed ingestion in the present study.

The hedonic properties of feed, including smell and taste, are known to activate pleasure sensations during feed ingestion, involving signals at the central level [21]. In teleost, the monoaminergic systems, which include serotonin- and dopamine-receptors, play a direct role in these mechanisms, inducing the activation of the brain reward system that may increase feed intake [24–26]. In the present study, the higher expression of serotonin 1A receptor (*5ht1a*) in both the caramel and ROT groups compared to the CTRL and coconut ones was coherent with the observed feed intake and fat accumulation results, indicating a state of higher satiety, possibly owing to the greater storage of energy reserves [50,51]. The positive modulation of the serotonin signaling pathway may contribute to the hedonic value of the diet and the desire to consume feed driven by reward sensation [52,53]. Differently, the significant dopamine receptor *drd3* downregulation in caramel group, respect to the ROT one, to values comparable to those observed in groups fed inert diets (CTRL and PG) and in the other group fed an attractive dietary stimulus (cheese), can be correlated with the reinforcement effects of a long-term exposure to a positive stimulus, related to the reward mechanisms [54]. For ROT and coconut groups, instead, it can be stated that the continuous rotation scheme or the long-term provision of a possibly not strongly perceived stimulus, respectively, were not able to trigger the reinforcement effects. These findings highlighted the importance of understanding the neurobiological mechanisms, underlying feeding behavior and the impact of different stimuli on feed intake and energy reserves. In fact, taken together, the results of the present study showed how the dietary implementation with caramel flavor was able to strongly increase the diet palatability, leading to improved feed intake and growth performance, supporting the role of feed's attractive sensory stimuli in regulating feeding motivation *via* central level-signals. Differently, the suboptimal results obtained by the ROT group can be mainly addressed to the inadequacy of the rotation scheme or to the alternation of caramel flavor with a lower powerful attractive flavor (cheese). In fact, fish from cheese group always showed comparable results to those from CTRL group. These findings are in accord to the preference observed by the zebrafish in the previous study [36]. Finally, the flavor priorly selected as repulsive (coconut) did not resulted as a strongly negative stimulus in this fish species, but rather could be classified as a not particularly perceived stimulus that was not able to significantly alter the overall perception of the basal diet, leading to comparable zootechnical performance to those observed in fish fed both CTRL and PG diets.

Conclusion

All the synthetic flavors tested did not affect the overall welfare of the fish. The provision of diet containing the F35 flavor (caramel odor) promoted a higher feed intake improving fish growth performance, confirming the attractive role of this flavor even in European seabass. In addition, the modulation of the monoaminergic systems suggested that the addition of caramel flavor in the diet was the most valuable solution compared to other ones tested, improving feed palatability and reward sensation. The present study demonstrated the promising effectiveness of synthetic flavors in advancing aquaculture feeding practices, paving the way for more sustainable and cost-effective progress in the aquaculture sector.

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Chapter 5

Innovative fish feeding strategies using dietary microencapsulated synthetic flavors: a focus on fish growth, welfare, and appetite stimulation in Gilthead sea bream (*Sparus aurata*) rearing

Abstract

Aquaculture is a rapidly growing sector that faces significant challenges in improving feeding efficiency and minimizing organic waste. A promising approach to enhance fish feed intake is the use of synthetic flavors as promising feed attractants, to stimulate fish feeding behavior. This study aimed to improve feeding practices in aquaculture by enhancing the incorporation of synthetic flavors into aquafeeds through a microencapsulation process. Two attractive flavors, cheese and caramel, were encapsulated in natural-based microcapsules (POP2 and POP3, respectively; 2.5%) to prevent flavor degradation and ensure their controlled release when dissolved to water. The effectiveness of the microencapsulated flavors as feed attractants was evaluated through a two-month feeding trial performed on juvenile Gilthead sea bream (*Sparus aurata*). The results indicated that the microencapsulation process ensured the rapid release of the flavors in water, with over 65% of the encapsulated flavor released within 3 minutes, demonstrating its potential to stimulate feeding behavior. Histological analysis of both liver and intestine showed no adverse effects, suggesting that the microencapsulated flavors did not affect the fish overall health status. However, no significant differences were observed in fish feed intake, growth, or feed conversion ratio among the experimental groups, despite the attractiveness of synthetic flavors previously demonstrated in other studies. Moreover, no significant differences were found in the gene expression of appetite-regulating pathways, taste receptors, or dopaminergic activity. The findings underscore the complexity of developing universal feed attractants and highlight the need for species-specific optimization in aquaculture nutrition research. Nevertheless, the results highlighted the potential of microencapsulation to improve flavor stability and bioavailability in aquafeeds. However, further studies are necessary to optimize the microencapsulation process, considering both concentration and releasing time of synthetic flavors, in order to develop tailored feed attractants that enhance feeding efficiency and contribute to sustainable aquaculture practices.

Introduction

Aquatic feeds are widely recognized as one of the most important protein sources for human nutrition. In an ever-expanding world population, estimated to reach 9.7 billion people by 2050, seafood consumption has more than doubled in the past 50 years, globally providing about 17 percent of animal protein [1]. Similarly, over the past three decades (1990-2020), the aquaculture sector has grown at an average annual rate of 6.7 percent, with aquafeeds being the dominant contributor to both its economic and environmental aspects. In fact, aquafeeds account for over 50 percent of the total aquaculture cost, largely due to low-efficiency feeding practices and to the source of ingredients used for their formulations [2–4].

Considering this latter aspect, the conventional fishmeal (FM) that is commonly used for aquafeed formulations, is nowadays a limiting factor for the industry. In fact, both its high unsustainability and costs make it not suitable as a sole source of protein in fish diets. At the same time, the inclusion within aquafeeds of alternative and more sustainable protein sources, has negatively affected their palatability [5,6]. In fact, although ingredients such as animal by-products (e.g., meat, bone, and poultry meal) [7,8], insects [9], and single-cell proteins of different origins (e.g., microalgae, yeasts, fungi, or bacteria) [10,11] have successfully replaced FM from the nutritional standpoint, the presence of off-flavors and bitter substances (e.g., anti-nutritional factors, alkaloids, chitin, mycotoxins) have impaired the feeds' overall attractiveness [5,6].

Fish farmers are compelled to overfeed the fish to ensure adequate intake, leading to a feed wastage which represents a direct financial loss for the industry. As a result, the release of organic matter into the waste flows causes eutrophication and imbalance of aquatic ecosystems, while the inadequate nutrient ingestion impairs the fish growth and health status, affecting farm's production yields [12,13].

Feed palatability currently remains a major challenge in aquaculture, requiring a crucial solution to improve the feeding strategy and reducing uneaten feed. Nowadays, numerous approaches exist to achieve a better feed efficiency of diet formulations. In particular, feed additives are substances which are included in trace amounts with different purposes. For instance, they are included within feed to facilitate ingredient dispersion or feed pelleting (emulsifiers, stabilizers, and binders), or to enhance feed ingestion by fish using feed attractants (FAs) [14]. In fact, by using FAs it is possible to modify both feed's flavor and taste, enhancing feed consumption by stimulating the fish's chemosensory capabilities [15,16].

Currently, the conventional FAs are mostly represented by natural resources, such as meal derived from mollusks, crustaceans, and small pelagic fish, posing however further unsustainability issues for the aquafeed sector [17]. In addition, the current alternative substances used as attractants only partially mitigates this problem, as attractive molecules such as L-amino acids, nucleotides, and nucleosides present limitations and disadvantages in their application [18]. In fact, the effectiveness of these substances is influenced by the type of molecules, as well as the concentration and the fish species considered, leading to controversial results [19]. In this regard, it is crucial to find innovative solutions to improve aquaculture feeding strategies, for a sustainable production while safeguarding natural resources.

Recent studies about the use of liquid synthetic flavors [20,21] provided important insights about the application of these substances as effective feed attractants. At the same time, however, their incorporation

method within fish diets has not been optimized. In fact, while their effectiveness in improving aquafeed palatability has been well-established, their incorporation methods required a daily addition to the feed. This is necessary to prevent both the flavor's leaching and deterioration, leading however to a time-consuming process unsuitable for industrial purposes. Thus, it becomes critical to explore alternative supplementation strategies to optimize flavors delivery, with a focus on their embedding implementation.

Microencapsulation technology can offer great potential for flavors incorporation within aquafeeds. This concept relies on the inclusion of a substance/flavor into a matrix, preventing its leaching and avoiding its direct exposure to the environment [22]. Several encapsulation techniques have been described, such as spray drying, spray chilling, extrusion coating, liposome entrapment, interfacial polymerization, solvent evaporation, solvent diffusion, nano-precipitation, salting-out, or electrohydrodynamic atomization [23]. Considering the type of wall materials, food-grade biopolymers can be used in the feed industry, including carbohydrates, gums, proteins, and lipids [24–27]. For instance, starch and natural gums are commonly used wall components to encapsulate active substances [23,28–30]. Microencapsulation in dry coating relies on the combination of different wall water-soluble materials, to improve the effectiveness of encapsulation systems, simultaneously enabling the flavors release in water. Once the flavor is encapsulated, the microcapsules containing flavor can be efficiently stuck to the feed, ensuring an effective and long-lasting incorporation.

The dissolved chemical stimuli released by microcapsules, can effectively stimulate fish to seek and ingest the feed, by activating their gustatory and olfactory senses, *via* the activation of olfactory and taste receptors [31]. In fact, feeding behaviors in teleost are primarily driven by sensory organs, and integrated by complex neuroendocrine signals between the brain and peripheral districts, which eventually regulate nutritional status and energy expenditure [32]. Neuropeptide Y has the strongest orexigenic role in regulating appetite at central level, while ghrelin, a hormone mainly produced by entero-endocrine cells of the gastrointestinal tract, regulates metabolism, modulates hormones release and stimulates feed intake [33]. Conversely, among the anorexigenic signals, leptin, produced in the liver, monitors nutritional balance eventually inducing satiety by upregulating the anorexigenic pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART), while downregulating orexigenic signals (mainly agouti-related peptide/AGRP and NPY) [34]. Among appetite-controlling systems, the fish brain's monoaminergic pathways play a crucial role in regulating feeding behavior [35–37]. Serotonin and dopamine are in fact known to influence feeding behaviors, cooperating with other appetite stimuli.

On this basis, the aim of the present study was to assess the effectiveness of dietary microencapsulated synthetic flavors on gilthead sea bream (*Sparus aurata*), as one of the most important species in Mediterranean aquaculture. The combination of synthetic flavors and natural-based microencapsulation processes applied to aquafeed formulation may offer great opportunities for an environment-friendly aquaculture obtaining high-quality products. By improving feeding practices through the use of upgraded/encapsulated flavors, it is possible to reduce feed wastage while simultaneously promoting the growth and welfare of the farmed species, thereby contributing to the blue bioeconomy concept of aquaculture.

Materials and Methods

Ethics

The feeding trial was conducted at the Institute of Marine Biology, Biotechnology and Aquaculture (IMBBC) of the Hellenic Centre for Marine Research (Heraklion, Greece). The experiments were authorized by the ethics committee of the region of Crete, Greece (license No EL91-BIObr-03) and were conducted following the guidelines established by the EU Directive 2010/63/EU.

Composition of microencapsulated synthetic flavors

Two attractive (cheese and caramel) flavors were produced by To Be Pharma S.r.l. (S. Egidio alla Vibrata, Teramo, Italy) following the same compositions reported in Conti et al. (2023). Subsequently, the flavors were encapsulated by STM Aquatrade S.r.l. (Castel Raimondo, Macerata, Italy) through a new technology called Co.M.E. (Coating Made Easy) within the +POP (Powder on Pellets) concept, a private project of STM Aquatrade S.r.l. According to Zarantoniello et al. (2024), the microcapsule (+POP) matrix wall was composed by Arabic gum (55%), starch (22%), and a lower percentage of cellulose, sodium ascorbate, and Vitamin E. The +POP composition is reported as relative abundance of each single chemical (first one more abundant, last one less abundant), and cannot be fully disclosed due to intellectual property protection. This encapsulation methodology prevents both the flavors leaching and degradation, simultaneously ensuring their deliverability to the feed through specific physic-chemical interactions which promote the dry microcapsules adhesion to the feed. Microcapsules quickly release their content *via* water dissolution, and 1 g of +POP can transfer 25 ppm of the carried flavor (corresponding to the 2.5% of the +POP composition).

UV–Vis absorption spectroscopy

To determine the time in which the flavor is released by microcapsules once in contact with water, a preliminary test was performed. Absorption spectra of both empty microcapsules and flavor-encapsulated +POP dissolved in water, were collected between 200 and 450 nm, with a concentration of 1 mg/mL, at every minute over the experiment and at room temperature (Jasco V-570 spectrophotometer). The samples were housed in a 1 cm path-length quartz cuvette.

Production of experimental diets

A commercial diet for gilthead sea bream (Zoonomi S.A, Greece) rich in land animal proteins and plant-based proteins, was used as control diet (CTRL). The available details of the control diet are described in Table 1. Then, three experimental diets were prepared: (i) POP1 diet, obtained by adding 5 g/kg feed of cheese flavor microcapsules (containing 2.5% of cheese flavor) to the control diet; (ii) POP2 diet, obtained by adding 5 g/kg feed of caramel flavor microcapsules (containing 2.5% of caramel flavor) to the control diet ; (iii) POP3 diet, obtained by adding 5 g/kg feed of empty microcapsules to the control diet.

After the addition to the feed, all the ingredients were mixed vigorously to ensure a uniform coating of the microcapsules to the feed pellets.

Table 1. Ingredients and proximate composition (g/100 g) of the experimental diets used in the present study.

CTRL diet	
Proximate composition (%)	
Crude protein[1]	46%
Crude fat[2]	16%
Crude ash	9%
Crude fiber	1.7%
Ca	1.3%
P	1.2%
Na	0.4%
Antioxidants	
BHA	30 ppm
BHT	40 ppm
Additives	
Vit A	11.000 UI/Kg
Vit D3	1.500 UI/Kg
Vit E	300 mg/Kg
Fe ($FeSO_4$)	150 ppm
Zn (ZnO)	37 ppm
Zn	18 ppm
$MnSO_4$ H_2O	38 ppm
Mn	9 ppm
$CuSO_4$ $5H_2O$	2 ppm
Cu	5 ppm
I [$Ca(IO_3)_2$] (Calcium iodate)	0.75 ppm
$C_5H_{11}NO_2Se$ (L-Selenomethionine)	0.3 ppm
Preservatives	
Citric acid	30 ppm

[1]Fish meal, wheat flour, poultry meal, sunflower meal, corn gluten, poultry blood meal, hydrolyzed feather meal, fermented soybean meal. [2]Fish oil, soybean oil.

Experimental design

Juvenile gilthead sea breams were obtained from the Institute of Marine Biology, Biotechnology and Aquaculture (IMBBC) hatchery of the Hellenic Centre for Marine Research (Heraklion, Greece). Three hundred fish were individually weighed (74.61 ± 0.7 g) after a mild anesthesia (phenoxyethanol, 150 ppm) and then randomly divided into 12 open circulation indoor tanks (500 L, 25 fish per tank), according with four experimental groups (in triplicate): (i) CTRL group, fish fed control diet; (ii) POP1 group, fish fed POP1 diet; (iii) POP2 group, fish fed POP2 diet; (iv) POP3 group, fish fed POP3 diet.

The duration of the feeding trial was two months, during which fish were hand-fed the experimental diets until apparent satiation, two times a day, 6 days a week. Pellets that remained unconsumed were daily siphoned and dried to determine feed intake. The experimental parameters throughout the whole experimental trial were water temperature, 19.9 ± 0.1 °C; salinity, 35 ppt; oxygen saturation, constantly over 80%; photoperiod of 12 h light/12 h dark. At the end of the feeding trial, all the fish were mildly anesthetized and individually weighed. In addition, samples from brain, liver, and intestine were collected from five fish per tank (15 per experimental group), after a lethal dose of anesthesia (phenoxyethanol, 500 ppm) and properly stored for further analyses. Fish were fasted 24 h prior organs sampling to reduce handling stress and ensure an empty gastrointestinal tract.

Zootechnical performance and somatic indexes

Data from biometry and feed intake were used to calculate the following zootechnical performance and somatic indexes:

Survival rate (%) = 100 × final number of fish / initial number of fish;

Weight gain (WG, %) = 100 × (FBW (final body weight, g) – IBW (initial body weight, g)) / IBW;

Specific growth rate (SGR, % day^{-1}) = 100 × *ln* (FBW / IBW) / number of days;

Daily feed intake (DFI, % body weight day^{-1}) = number of days × total dry feed intake (g) × 100 / ((IBW + FBW) × 0.5);

Feed conversion ratio (FCR) = total dry feed intake (g) / weight gain (g);

Hepatosomatic index (HSI) = 100 × liver weight (g) / body weight (g).

Histology

Samples of liver and distal intestine were collected from 5 fish per tank (15 per experimental group) and fixed in formaldehyde/glutaraldehyde solution (NaH_2PO_4–H_2O + NaOH + Formaldehyde (36.5%) + Glutaraldehyde (25%) + H_2O) at 4 °C for 24 h. Subsequently, samples were washed three times in 70% ethanol (15 min each) then stored at 4 °C in the same ethanol solution. Samples were dehydrated using increasing gradation of ethanol solutions (80, 95, and 100%), then washed with xylene (Bio-Optica, Milano, Italy), and finally embedded in paraffin (Bio-Optica) blocks. A microtome (Leica RM RTS, Nussloch, Germany) was used for cutting paraffin sections of 5 µm in thickness at 50 µm intervals, which were subsequently stained with Mayer hematoxylin and eosin Y (Merck KGaA). Finally, the tissues physiological architecture and/or the occurrence of inflammatory events were assessed in both hepatic parenchyma and intestinal tract.

Considering liver, the fat fraction percentage (FF%) was quantified on liver sections without blood vessels or bile ducts using ImageJ software (ver. 1.54d), setting a homogeneous threshold value. The FF% was expressed as the percentage of the area occupied by fat on the total hepatic parenchyma (images acquired at 400×).

Considering the distal intestine, histological indexes were performed on three transversal sections per fish (15 specimens per experimental group). Specifically, the height of all the undamaged and non-oblique mucosal folds was measured using ZEN 2.3 (Zeiss, Oberkochen, Germany). The scores for the different histopathological indexes were assigned as follows: (i) mucosal folds fusion: + = 0–3 observations per section, ++ = 3–10 observations per section, +++ ≥ 10 observations per section; (ii) inflammatory influx: + = scarce lymphocyte infiltration, ++ = moderate lymphocyte infiltration, +++ = diffused lymphocyte infiltration.

Molecular analyses

Total RNA extracted from brain and distal intestine samples of 3 fish per tank (in triplicate), using RNAzol (Sigma-Aldrich, St. Louis, MI, USA), were reverse transcribed into cDNA using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). To perform the reverse transcription, a DNase treatment (10 IU at 37 °C for 10 min; MBI Fermentas, Milano, Italy), was performed on total RNA to digest genomic DNA. Moreover, both an agarose gel (1%) electrophoresis, running 1 µg of total RNA stained with GelRed, and quantification using NanoPhotometer P-Class (Implen, München, Germany), were performed to check total RNA integrity and purity, respectively.

The relative mRNA abundance was determined by real-time quantitative PCR (qPCR) reactions, performed in an iQ5 iCycler thermal cycler (Bio-Rad, Hercules, CA, USA) on a 96-well plate. Analyses were performed using the parameters and reagents reported in Zarantoniello et al. (2024). Two reference genes, beta-actin (*β-actin*) and 18S ribosomal protein (*18s*), were used to standardize the results and the relative quantification of gene involved in taste system (taste receptor, type 1, *t1r1*; taste receptor, type 1, member 2a, *t1r2a*; taste receptor, type 1, member 2b, *t1r2b*; taste receptor, type 1, member 3, *t1r3*) appetite regulation (neuropeptide Y, *npy*; ghrelin, *ghrl*), and brain reward system (dopamine receptor D2, *drd2*) was determined on samples from target organs.

Particularly, (i) *npy*, and *drd2*, were tested in brain samples, while (ii) *ghrl, t1r1*, *t1r2a*, *t1r2b*, *t1r3*, were tested in intestine samples. The primer efficiency was checked for all the primer sets included in the study. The RT-qPCR reactions were performed with 1 µL of 1:10 diluted-cDNA, 5 µL of fluorescent intercalating agent (2× concentrated iQ ™ Sybr Green, Bio-Rad, Milano, Italy) and 0.3 µM of both forward and reverse primer. The thermal profile was 3 min at 95 °C, then 45 cycles of 20 s at 95 °C, 20 s at the melting temperature specific for each primer (reported in Table 2), and 20 s at 72 °C. Two no-template controls (NTCs) were added in each run to guarantee the absence of contamination in the Master Mix. The mRNA levels of target genes were calculated using the geometric mean of two stably expressed reference genes using the Bio-Rad CFX Manager 3.1 software. The qPCR data were processed using iQ5 optical system software version 2.0 (Bio-Rad), including GeneEx Macro iQ5 Conversion and genex Macro iQ5 files. Amplification products were sequenced, and homology was verified.

Gene	Forward Primer (5'–3')	Reverse Primer (5'–3')	A.T. (°C)	NCBI ID
npy	GGGTTGTTTTCAGGACCAAG	CGCAGAAAGGAGAAGAGCAA	58	XM_051426243
ghrl	GGAAAGTCTTCCAGGGTCGG	CGCATAGTCCTCTTCTGTCATGGAG	59	XM_030418498.1
drd2	GAATGAGCTGCGAGGTGAA	CGTGGGTTGTTGGTATGAGA	58	XM_051411236
t1r1	CTGATGCAAGCCATGAGATTTGCTG	CCAATCAAAGCCAATCCTCTCTGA	59	XP_030277517
t1r2a	AGGAGTGATTGTTGAGATCATTCTGC	ACTCAATGAGAGCTTCAGCAGTCAAT	59	XP_030278006
t1r2b	CACTTACATAAGCACTGAAACACGGACTG	CATCTATGTTAAGGAAAGAAACCCAGCG	58	XP_030278002
t1r3	GGAGACATTATCCTCGGAGGGCTCT	TTCCGAGCTATAAGGACCGATCACGGCT	59	XP_030274769
b-actin (hk)	GGTACCCATCTCCTGCTCCAA	GACGTCGCACTTCATGATGCT	60	AJ_537421
18s (hk)	AGGGTGTTGGCAGACGTTAC	CTTCTGCCTGTTGAGGAACC	60	XM_051390998

Table 2. Primer sequences used in the present study, annealing temperature (A.T.), and NCBI IDs.

Statistical analysis

The tanks were used as the experimental unit for data related to survival rate and zootechnical performance, while fish were considered the experimental unit for all the remaining analyses. Normality and homoscedasticity were checked for all the data through Shapiro–Wilk and Levene's tests, respectively. Data were then analyzed using a one-way ANOVA followed by Tukey's multiple comparison test. Significance was set at $p < 0.05$, and results were expressed as mean ± standard deviation (SD). The statistical software package Prism-8 (GraphPad Software, San Diego, CA, USA) was used for the data analysis.

Results

Flavor-release in water

The UV absorbance of the flavored-microcapsules dissolved in water indicated that, after 3 minutes, over 65% of the total encapsulated flavor was released by microcapsules, as reported in Figure 1. Moreover, the characteristic absorption bands observed in the absorption spectra can be related only to the flavor released in water, since the empty-microcapsules spectra did not display specific band at any wavelength considered.

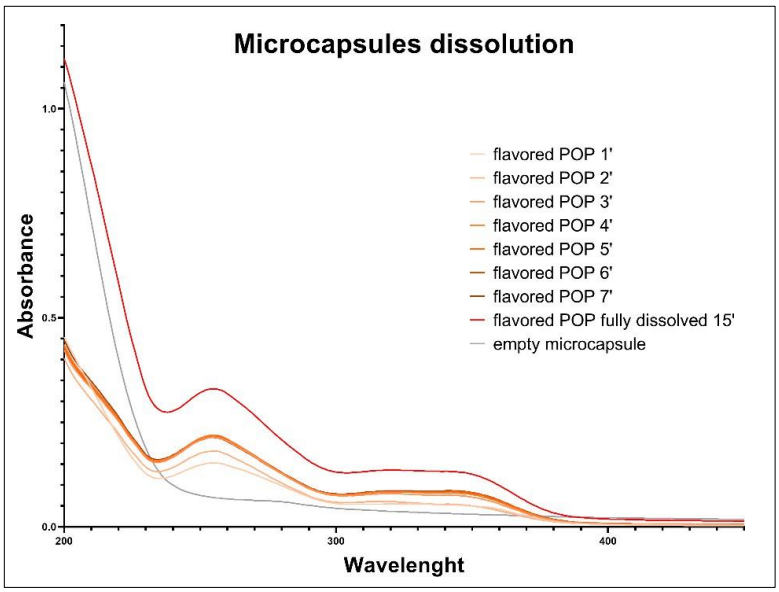

Figure 1. UV–Vis absorption spectra of both empty microcapsule (grey) and encapsulated-flavor, for each minute of dissolution in water, and absorption spectra of totally dissolved encapsulated-flavor (red).

Zootechnical performance and somatic indexes

At the end of the feeding trial, survival rate was 100% in all experimental groups, and fish duplicated their initial weight (143.6 ± 0.6 g) during the 62-day feeding trial (Table 3).

As reported in table 2, specimens from all the different experimental groups showed no significant differences in all the zootechnical indices analyzed (final body weight, weight gain, specific growth rate, daily feed intake), as well as in the hepatosomatic index (HSI).

	CTRL	POP1	POP2	POP3	*P-Value*
Survival %	100	100	100	100	-
IBW (g)	74.74	74.82	73.82	75.07	0.8671
FBW (g)	144.20	145.51	139.56	144.97	0.1541
WG (%)	93.05	94.50	89.09	93.14	0.6141
SGR (% day^{-1})	1.06 ± 0.05	1.07 ± 0.05	1.03 ± 0.04	1.06 ± 0.03	0.6453
DFI (% BW day^{-1})	1.62 ± 0.10	1.60 ± 0.09	1.56 ± 0.06	1.67 ± 0.03	0.4518
FCR	1.25 ± 0.04	1.22 ± 0.05	1.24 ± 0.01	1.28 ± 0.03	0.3966
Somatic indices					
HSI (%)	0.17 ± 0.02	0.19 ± 0.03	0.18 ± 0.06	0.18 ± 0.03	0.4687

Table 3 - Zootechnical performance and somatic indices of gilthead sea bream fed the experimental diets. Values are shown as mean ± SD (n = 3). Abbreviations: IBW, initial weight; FBW, final weight; WG, weight gain; SGR, specific growth rate; DFI, daily feed intake; FCR, feed conversion ratio; HSI, hepatosomatic index.

Histology

With regard to the liver and intestine of specimens from the different experimental groups, no significant alterations in tissue's morphology or signs of inflammation were detected (Figure 2). In particular, considering distal intestine, no pathological alterations were observed in all the experimental groups, including a scarce lymphocytes infiltration, absence of supranuclear vacuoles, and isolated episodes of mucosal folds fusion (Table 4). Moreover, no significant differences were detected in terms of both mucosal fold height and submucosa width.

Considering liver, a physiological structure of hepatic parenchyma was observed in all the experimental groups, characterized by hepatocytes with abundant intracytoplasmic accumulation of fat accumulation. Corroborating this, no significant differences were detected in the statistical quantification of the fat fraction percentage among groups, calculated on liver sections (53.33 ± 10.74, 58.30 ± 7.98, 59.50 ± 6.31, 54.90 ± 8.64, for CTRL, POP1, POP2, and POP3, respectively; $p > 0.05$).

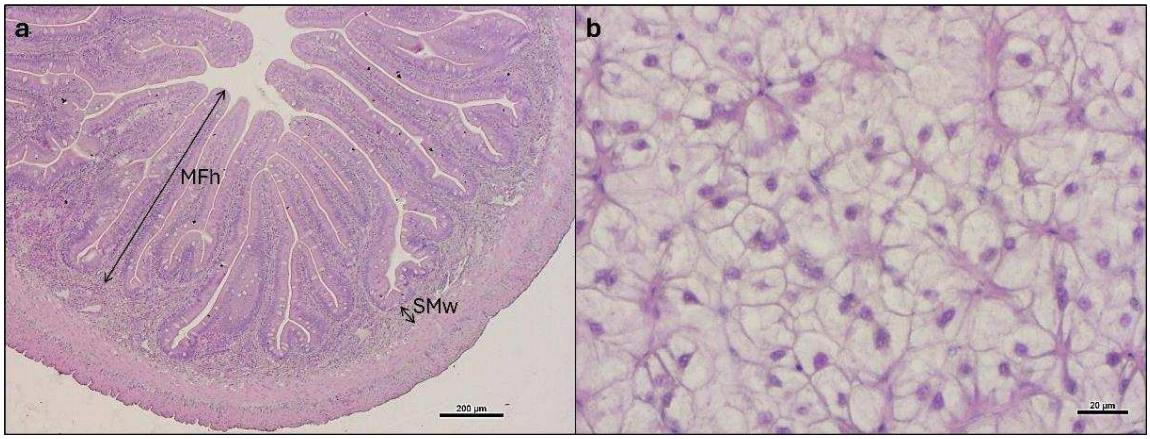

Figure 2. **Histological evaluation of liver and distal intestine.** (**a**) Example of histomorphology of distal intestine from gilthead sea bream fed the POP2 diet with measurement criteria for submucosa width (SMw) and for the mucosal fold height (MFh). (**b**) Example of histomorphology of liver from gilthead sea bream fed the POP1 diet. Scale bar: 200 µm.

	CTRL	POP1	POP2	POP3
Mucosal fold height	741.1 ± 31.4	794.5 ± 110.2	712.2 ± 54.3	756.1 ± 73.7
Submucosa width	31.3 ± 2.5	32.2 ± 1.9	30.8 ± 1.9	32.3 ± 2.5
Mucosal fold fusion events	+	+	+	+
Basal inflammatory influx	-	-	-	-
Supranuclear vacuole	-	-	-	-

Table 4. Histological indexes measured in the distal intestine of gilthead sea bream fed experimental diets. Values for mucosal fold height and submucosa width are shown as mean ± SD (n = 30).

Real-Time PCR

Appetite. Considering both *npy* and *ghrl* relative gene expression (Figure 3), no significant differences were detected among experimental groups.

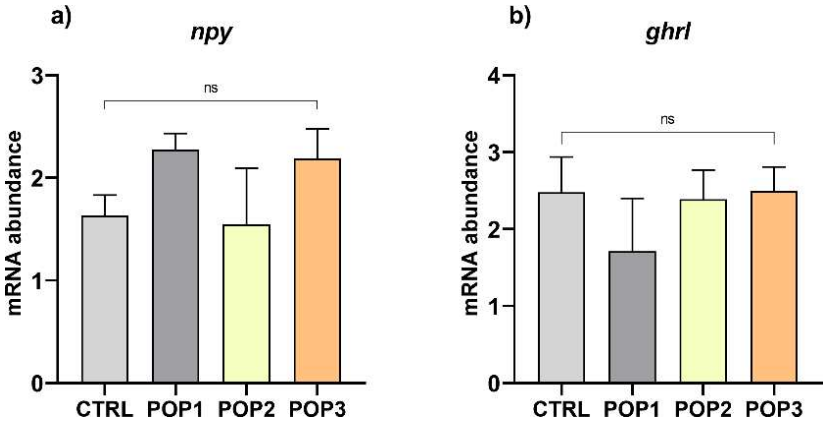

Figure 3 - Real-time qPCR results. Relative mRNA abundance of appetite-related markers, analyzed in gilthead sea bream brain (*npy*) and intestine (*ghrl*) samples. (**a**) *npy*, (**b**) *ghrl*. Results are expressed as mean + SD (n = 5). ns, no significant differences.

Taste receptors. Considering the relative gene expression of *t1r1*, *t1r2a*, *t1r2b*, *t1r3* (Figure 4), no significant differences were detected among groups.

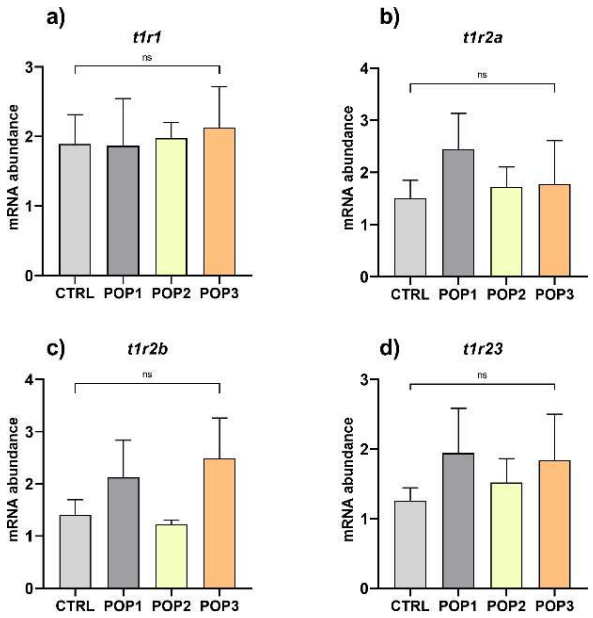

Figure 4 - Real-time qPCR results. Relative mRNA abundance of markers related to gustatory systems, analyzed in gilthead sea bream intestine samples. (**a**) *t1r1*, (**b**) *t1r2a*, (**c**) *t1r2b*, and (**d**) *t1r3*. Results are expressed as mean + SD (n = 5). ns, no significant differences.

Dopaminergic activity. Considering *drd2* relative gene expression (Figure 5), no significant differences were detected among experimental groups.

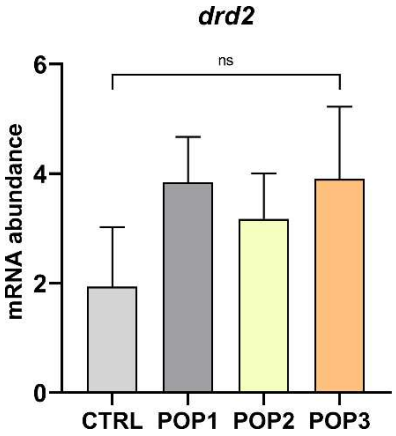

Figure 5 - Real-time qPCR results. Relative mRNA abundance of marker related to dopaminergic system (dopamine D2 receptor, *drd2*), analyzed in gilthead sea bream brain samples. Results are expressed as mean + SD (n = 5). ns, no significant differences.

Discussion

Improving the feeding practices of aquaculture is of crucial importance to safeguard the sector's economy and the environment. Stimulating fish to ingest a proper amount of nutrients by using effective feed attractants, can minimize the feed wastage while improving production yields. Attractive synthetic flavors fully meet this purpose, as previous studies [20,21] in zebrafish have demonstrated their promising role as feed attractants. The method for incorporating synthetic flavors into aquafeeds, however, has not been optimized yet, as they are primarily added in liquid form. This approach leads to a flavor degradation over time, due to their environmental exposure, and thereby requiring the flavors' daily addition to the pellet. Considering this important limitation especially for industrial purposes, the aims of the present study were: (i) improve the incorporation method of liquid synthetic flavors using a microencapsulation process and (ii) test their effectiveness as encapsulated feed attractants through a feeding trial on gilthead sea bream. This innovation allowed the encapsulation of liquid flavors within the natural-based microcapsule walls, which can easily adhere to any type of aquafeed. Subsequently, upon contact with water, the microcapsules stuck to the feed can quickly release the flavor, and thereby the feeding stimulus. This aspect was easily demonstrated by the absorption spectra obtained within the first minutes of flavor dissolution in water, during which over 65% of the microcapsules' content (the flavor) was released and dissolved in water. In this way, flavors can trigger a rapid response from the fish when the feed is provided, enhancing their feeding activity and encouraging them to consume the feed quickly. At the same time, when the feed is stored, the flavor can maintain its organoleptic qualities avoiding degradation [38].

Besides this first consideration, the welfare of the farmed species is of paramount importance during farming. For that reason, the health status of target organs involved in digestion and metabolism must be evaluated when introducing novel additives into feed formulations. In the present study, all the tested diets had no adverse effects on the physiological structure of liver and intestine, as demonstrated by the histological analysis.

On the other hand, despite previous studies demonstrated the strong attractiveness of both cheese- and caramel-flavors throughout the zebrafish life cycle [20,21], no marked responses were observed during the 2-month feeding trial in gilthead sea bream. In fact, the effectiveness of attractive flavors in enhancing fish feeding response is typically reflected in increased feed ingestion. In the present study, daily feed intake did not evidenced differences over the whole experimental period, despite a slight increase in POP6 group. Corroborating this, the zootechnical parameters such as weight gain, growth rate and feed conversion ratio, remained consistent in all the experimental groups, having fish ingested a similar amount of nutrients (and thus having the same available energy to invest for growth).

Feed attractants action on fish is mainly exploited by modulating both olfaction and taste receptors, which in turn can stimulate appetite-regulating signals (e.g., orexigenic NPY) and brain reward pathways [35–37]. In fact, the expression of taste receptors, along with orexigenic and dopaminergic signals, is deeply influenced by the consumption of palatable feed [31,39]. These neuro-endocrine pathways are activated during the detection and ingestion of feed, playing a key role in shaping the overall fish feeding response. More specifically, the activation of the taste receptors can in turn stimulate the NPY expression at the central level,

increasing the appetite sensation [31,40]. Finally, the high palatability of the diet influences the dopaminergic activity in the brain, triggering the pleasure sensation and reinforcing the feeding behavior [36]. Previous studies clearly demonstrated that the synthetic cheese- and caramel-flavor tested effectively activated these mechanisms in zebrafish [20,21]. However, in this study their effectiveness may have been reduced during the microencapsulation process, resulting in a less pronounced effect on feeding stimulation. Moreover, although zebrafish is a well-validated model species, Gilthead seabream has its own unique ecology and feeding behaviors, and the attractiveness of many feed attractants is often species-specific and concentration-dependent [18,41]. Finally, the activation of the appetite-signals, is influenced by various pathways, and a 2-month period is likely too short to elicit a strong response to a novel diet, at least in *Sparus aurata* [40,42].

Conclusions

Despite the limitations which could have affected the above-described outcomes, this preliminary study highlighted the potential of the microencapsulation process to improve the feeding practices in aquaculture. Liquid synthetic flavors incorporation method can be effectively implemented, enhancing the flavors' stability and bioavailability in aquafeeds, and this approach paves the way for developing better-formulated diets. However, there is a critical need for comprehensive testing of synthetic flavors to develop tailored feed attractants for each species of commercial interest. Identifying optimal concentrations of these attractants will be essential to maximize their effectiveness and improve the overall feeding response, ultimately leading to more efficient and sustainable aquaculture practices.

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Chapter 6

Final remarks and conclusion

The present PhD thesis aimed to identify different synthetic flavors which can display a potential role as feed attractants within fish diets. In particular, the utilization of these flavors was specifically selected due to the current reliance of the feeding stimulants on natural resources and their inconsistent effects, which vary greatly in effectiveness. In fact, traditional feed attractants are either derived from marine sources or represented by a limited number of molecules (e.g., amino acids), often unsustainable and with unpredictable attractiveness. This contributes to both environmental and economic challenges in aquaculture, as it leads to feed wastage due to inadequate nutrient intake. Optimizing feeding practices is of paramount importance for improving production efficiency while preserving sustainability in fish farming.

Synthetic food flavors, commonly used in human food production, can offer a novel, more sustainable and consistent alternative to traditional feed attractants. Produced through standardized processes, they present a reduced environmental and economic footprint, allowing for precise replication of specific stimuli – whether smell or taste – ensuring a more effective and reliable feeding response by the fish. Additionally, synthetic flavors can be tailored to mimic virtually any odor, enabling a better match to the dietary preferences of the different farmed species.

Due to the paucity of information about the use of synthetic flavors in the aquafeed sector, the first trial of the present PhD thesis was mainly focused on evaluating different synthetic flavors with a potential attractive effect for fish. In this regard, forty different commercial synthetic flavors were initially assessed through a behavioral preference test, performed on zebrafish larvae, in which each flavor was tested singularly. The behavioral test allowed the identification of two attractive (caramel and cheese) and one repulsive (coconut) flavors, which were selected to be used in the following analyses of the study. More specifically, in the first section, the selected flavors were subsequently added to a specific zebrafish diet which was administered to the fish from the larval to the juvenile stage. None of the synthetic flavors tested affected the overall health of the fish. In fact, histological and molecular analyses in the liver and intestine revealed no alterations in tissue's structure and histopathological indexes, or inflammation events, at either developmental stage. On the other hand, the provision of diets containing attractive flavors (cheese and caramel), resulted in an increased growth rate in both larvae and juveniles. Moreover, fish showed higher appetite stimulus and feed ingestion, validating the preference of fish for the attractive diets. Finally, the higher feed ingestion led to a higher lipid storage, beneficial for early life stages during energy investment. The modulation of the fish dopaminergic activity suggested the influence of the attractive diets on fish brain reward system, particularly suggesting the selection of the caramel-flavored diet as the most valuable solution for maintaining its long-lasting effect over the whole experimental period. Taken together, the findings from the first section offer valuable insights into the potential use of attractive synthetic flavors in aquafeed production, paving the way for more sustainable and

economically beneficial opportunities in the aquaculture industry. Nevertheless, further research is needed to test the same flavors on adult fish, evaluating their effects over the whole life cycle, with a particular focus on reproduction.

Since fish life phases may display behavioral differences related to their developmental stages, the second section was focused to test the effects of the same cheese- and caramel-synthetic flavors during a six-month feeding trial on zebrafish (from larvae to adults). The addition of the solvent alone (Propylene Glycol) to the control diet did not show any effects on fish physiological responses, while the attractive synthetic flavors tested confirmed their attractiveness also in fish exposed over a long-term period. In fact, the inclusion of cheese- or caramel-flavored diets resulted in increased feed ingestion, driven by appetite and olfactory stimuli, which in turn were influenced by the hedonic properties of the feed. The provision of attractive diets positively promoted fish reproductive performances and growth rate, enabling the fish to invest to a greater extent in this high-metabolically demanding process. The outcomes highlighted the promising role of synthetic flavors in enhancing fish feeding strategies. Using these next generation of feed additives is possible to reduce the reliance on traditional feeding stimulants, as they are produced through standardized processes that ensure consistent attractiveness in aquafeeds. The use of synthetic flavors as attractants is in fact reliable throughout the life cycle of a model species like zebrafish, although further research is needed to evaluate their effectiveness in species of commercial interest. In fact, the fish feeding behavior can greatly vary in accordance with the ecology and biology of different species, their developmental stage, and environmental conditions. Formulating a tailored diet is of paramount importance to ensure the farm's production yield without affecting fish welfare.

In light of this, the third section aimed to test the attractiveness of the same cheese- and caramel-synthetic flavors on European seabass (*Dicentrarchus labrax*), during a 90-day feeding trial, evaluating the fish physiological responses. The provision of diet containing caramel-synthetic flavor promoted a higher feed ingestion which in turn promoted fish zootechnical performances. The attractive role of this flavor was thereby confirmed even in European seabass, without affecting welfare. In addition, the modulation of the monoaminergic systems suggested that the dietary addition of caramel flavor was the most valuable solution compared to other ones tested, stimulating reward pathways providing a more pronounced sensory stimulation and potentially yielding greater improvements in feed attractiveness and consumption. Assessing the efficacy of synthetic flavors as feed attractants for carnivorous fish species of commercial value is essential, considering the limitations of existing aquafeed formulations. This investigation is crucial for advancing feeding practices and optimizing nutritional management within the aquaculture industry, potentially leading to more efficient and sustainable production methods.

The fourth section introduced a novel technology for incorporating synthetic flavors into aquafeeds. During this phase, both cheese and caramel flavors were micro-encapsulated, embedding the liquid flavors within natural-based microcapsules. This technique ensured that the flavors adhered to the aquafeed and were released quickly upon contact with water. Following this process, both the micro-encapsulated cheese and caramel flavors were added to a diet for gilthead sea bream (*Sparus aurata*) and tested over a two-month feeding trial

to assess the fish's physiological responses.

Although previous studies in zebrafish demonstrated the attractiveness of both cheese- and caramel-flavor, their effectiveness may have been compromised during the microencapsulation process in this phase, leading to a diminished effect on feeding stimulation. Additionally, the efficacy of feed attractants is often species-specific and concentration-dependent, and these aspects may have influenced the effectiveness of these flavors in gilthead sea bream. Despite these limitations, this final section highlights the potential of microencapsulation technology to enhance feeding strategies in aquaculture. Upgrading liquid synthetic flavors through microencapsulation process can significantly improve their stability and bioavailability within aquafeeds, offering a promising approach for the development of more optimized diets.

Overall, the outcomes obtained in the present PhD thesis highlighted the effectiveness of synthetic flavors as a novel class of feed additives able to enhance the fish feeding response across different species and developmental stages. Through the collaboration with To Be Pharma S.r.l., it was possible to identify and successfully validate the efficacy of liquid synthetic flavors as feed attractants. In the first stages of this PhD thesis, an initial array of synthetic flavors demonstrated their effectiveness in enhancing growth and feed intake in zebrafish (an omnivorous model species), and European sea bass (a carnivorous species). Importantly, this successful transfer of the outcomes to commercially valuable species further confirmed the broad applicability of synthetic flavors across different feeding ecologies and behavioral traits.

Building on these findings, a further collaboration with To Be Pharma S.r.l. and STM Aquatrade S.r.l., enabled the development of a microencapsulation process aimed at implementing the feeds formulation. The microencapsulation process was designed to improve the suitability of liquid synthetic flavors for industrial applications, allowing for easier incorporation into aquafeeds while mitigating both their environmental exposure and degradation over time, protecting their quality. Microencapsulation also offers additional advantages, such as controlling the release of flavors in water and ensuring homogenous distribution within feed matrices. The resulting microencapsulated product was designed to meet the practical demands of aquaculture farming, facilitating efficient feed formulation and administration. Such innovations are critical for ensuring the long-term efficacy of feed additives in practical aquaculture settings, where environmental variables, such as water quality, temperature, and farming practices, can influence feed stability.

To rigorously assess the performance of the microencapsulated flavors as feed attractants, the second aim of the present PhD thesis was to perform experiments under farm-like conditions. This was achieved through a collaboration with a leading research center, the Hellenic Centre for Marine Research (HCMR), as part of an Aquaexcel European Project. The experimental design simulated grow-out conditions typical of intensive aquaculture, providing a relevant platform to evaluate the practical utility of microencapsulated feed attractants. In particular, the microencapsulated flavors were tested on gilthead sea bream, a commercially important species. However, the outcomes of this final stage did not yield the expected results, as no significant response in fish feeding behavior or growth performance were observed. This lack of effect may stem from several biological and technical factors. Firstly, the response to feed attractants is known to be highly species-

specific, with different species exhibiting varying sensitivities and preferences for chemical cues, influenced by their sensory systems, feeding behaviors, and ecological niches. Moreover, the concentrations of attractants, which proved effective in earlier trials with zebrafish and European sea bass, may not have been optimal for gilthead sea bream. Finally, the microencapsulation process itself, while protecting the flavors, may have altered their release dynamics or bioavailability, reducing their overall effectiveness in stimulating feeding.

Despite these limitations, this innovation holds significant potential for the aquafeed industry, enabling more precise feed formulations during key phases such as growth and reproduction. In fact, by promoting optimal nutrient intake, synthetic flavors help maintain fish welfare, supporting growth and energy investment in high metabolically demanding processes. This, in turn, improves production yields, product quality, and reduces feed wastage, offering economic and environmental benefits for fish farms. Finally, the utilization of synthetic flavors reduces the reliance on natural ingredients, simplifying both feed manufacturing and farming operations while reducing environmental footprint.

The new scientific knowledge presented in this PhD thesis can serve as a foundation for future research, potentially expanding the range of attractive synthetic flavorings by investigating their effects on different farmed species. The findings obtained underscore the complexity of developing universal feed attractants and highlight the need for species-specific optimization in aquaculture nutrition research. Further investigations are warranted to refine the formulation and delivery of these microencapsulated products, taking into account the specific sensory and physiological characteristics of target species to enhance their application in commercial aquafeeds. This approach could lead to the development of a diverse array of species-specific flavors, offering to the aquafeed industry a wider choice of tailored feed additives for optimized feeding strategies in any scenario.

Chapter 7

Integrating farming strategies to minimize the environmental impact of aquafeeds

Efficient fish farming requires maximizing the overall level of production, while minimizing feed input. In order to reduce the environmental impact of aquafeeds, apart from promoting higher feed consumption, additional aspects such as the implementation of diet formulations have to be considered. In fact, solid waste mainly originates from uneaten and/or spilled feed by the fish and from the feces excreted. The quantity and quality of this waste depend on different factors, including intake, digestion and metabolism of dietary compounds. Therefore, strategies to reduce the impact of aquaculture waste has to focus on feed composition, as well as on both feeding and farming strategies, with two key approaches: (i) improving the nutrient retention ability of the fish and (ii) enhancing waste removal efficiency.

Considering the first aspect, it can be achieved through optimized diet formulation, which leads to lower feed conversion ratios (higher efficiency). The FCR measures the efficiency with which animals convert feed into meat and other food products and is calculated as the ratio of feed intake to weight gain. Factors such as the digestibility of the ingredients and nutrient composition of diets significantly affect this index, since solid waste is mainly composed of undigested starch and fiber (especially from plant-based diets). Consequently, reducing waste discharge in aquaculture can be managed by using highly digestible diets.

However, despite these considerations, the application of highly digestible feeds cannot completely eliminate waste production, as digestion in fish has inherent limitations, leaving a fraction of the feed undigested and excreted as feces. Therefore, addressing this issue through effective solid waste removal can be another critical strategy to reduce the environmental impact of aquaculture wastewater.

Polyculture (or multitrophic aquaculture), which involves the production of different aquatic species in the same production system, offers a promising approach to decrease production costs, increase productivity and reduce waste output. One of the innovative solutions within this framework is integrated multitrophic aquaculture (IMTA), which balances environmental sustainability, economic diversification, and social acceptability. This practice integrates the cultivation of fed species (e.g., finfish) with organic extractive species (e.g., shellfish) and inorganic extractive species (e.g., seaweed), creating a balanced ecosystem management approach, tailored to site-specific needs, operational limits, and food safety standards.

For instance, seaweed and mussels thrive on nutrients from wastewater discharged by intensive fish farms, thereby reducing the environmental load. Additionally, fish waste can be re-used within aquaculture systems to produce valuable byproducts such as vegetables or bacteria. Nitrogen (N) and phosphorus (P) released into the water, especially in extensive and semi-intensive systems, can effectively be converted by phototrophic and heterotrophic organisms. Algae-based wastewater treatment is a well-established method to remove these

nutrients, converting dissolved waste into harvestable products. Algae can either be directly consumed by fish or used as an ingredient in fish feed, significantly improving nutrient retention, such as the ‘protein/calorie efficiency ratio’ of the diet, and enhancing overall feed efficiency.

Chapter 8

Appendix

Dissemination of results

Scientific publications

Conti, F. *et al.* (2023). The Application of Synthetic Flavors in Zebrafish (*Danio rerio*) Rearing with Emphasis on Attractive Ones: Effects on Fish Development, Welfare, and Appetite. Animals, 13(21), 3368. https://doi.org/10.3390/ani13213368 (Chapter 2).

Conti F. *et al*. (2024). The Promising Role of Synthetic Flavors in Advancing Fish Feeding Strategies: A Focus on Adult Female Zebrafish (*Danio rerio*) Growth, Welfare, Appetite, and Reproductive Performances. Animals, 14(17), 2588. https://doi.org/10.3390/ani14172588 (Chapter 3).

Contribution to scientific conferences

Oral communications

Conti, F., *et al*. A new set of feed additives to promote fish feed intake and welfare in aquaculture: a comparative study on zebrafish (*Danio rerio*) larval and juvenile stage. *Eas Aquaculture Europe 2023* – Vienna, Austria (Chapter 2).

Conti, F., *et al*. Effects of dietary administration of synthetic flavors on zebrafish (*Danio rerio*) growth, welfare, appetite stimulation and reproductive performances. *International Conference of Innovative Aquaculture 2024* - Gorgan, Golestan Province, Iran (Chapter 3).

Conti, F. *et al*. The use of synthetic feed attractants in zebrafish (*Danio rerio*) diets: effects on growth, welfare, appetite stimulation, and reproductive performance. *31st Conference of European Comparative Endocrinologists, 2024* – Ancona, Italia (Chapter 3).

Poster

Conti, F. *et al*. Behavioral responses to different feed additives in Zebrafish (*Danio rerio*) larvae: a preliminary study. *Eas Aquaculture Europe 2022* - Rimini (Italia) (Chapter 2).

Other publication

Conti, F. *et al*. Percorsi di dottorato di ricerca: opportunità di innovazione e crescita professionale per studenti ed aziende. Il Pesce, N. 4, 2022.