Universitat Autònoma de Barcelona

IMPROVEMENT OF THE HEALTH AND CONDITION OF FISH MUCOSAL TISSUES THROUGH FUNCTIONAL DIETS IN AQUACULTURE:
Phytogenics as additives for aquafeeds

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MEJORA DE LA SALUD Y CONDICIÓN DE LAS MUCOSAS EN PECES MEDIANTE EL USO DE DIETAS FUNCIONALES EN ACUICULTURA: FITOGÉNICOS COMO ADITIVOS PARA ALIMENTOS ACUÍCOLAS

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"IMPROVEMENT OF THE HEALTH AND CONDITION OF FISH MUCOSAL TISSUES THROUGH FUNCTIONAL DIETS IN AQUACULTURE: PHYTOGENICS AS ADDITIVES FOR AQUAFEEDS"

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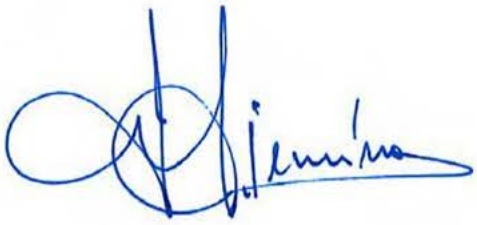

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"A perseverança é a mãe da boa sorte. Quanto maiores as dificuldades a vencer, maior será a satisfação."

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ABSTRACT

Aquaculture growth will unavoidably involve the implementation of innovative and sustainable production strategies, being functional feeds among the most promising ones. A wide spectrum of phytogenics, particularly those containing terpenes and organosulfur compounds, have been gaining increasing interest in aquafeeds, due to their growth promoting, antimicrobial, immunostimulant, antioxidant, anti-inflammatory and sedative properties. Although the impact of phytogenics upon fish mucosal immunity has been extensively evaluated, most of the studies fail in addressing the mechanisms underlying their pharmacological effects. Under this context, the set of studies gathered in this thesis aims to provide insights on how fish mucosal tissues are immunomodulated by phytogenics, in particular by the administration of a microencapsulated feed additive composed by garlic essential oil, carvacrol and thymol. A holistic approach integrating both key performance indicators and the transcriptional immune response of three selected mucosal tissues – gill, intestine and skin – was applied in one of the most important marine fish species farmed in the Mediterranean Sea, the gilthead seabream (*Sparus aurata*). The overall analysis of the results indicated that the dietary supplementation of garlic, carvacrol and thymol promote the gilthead seabream mucosal innate immunity and the mucus protective capacity, decreasing the mucosal tissues susceptibility to be infested by parasites and colonized by pathogenic bacteria, without compromising somatic growth or microbiota homeostasis. Therefore, the strategy evaluated is an effective and safe tool to be used in functional diets for aquaculture. Besides solving scientific questions, the overall results and added knowledge obtained from this industrial thesis aim to drive the development and consolidation of new nutritional tools that may be used to hamper emergent disease outbreaks related to the growth in intensive production systems and the increasing pressure of climate change.

Keywords: Functional diets, phytogenics, garlic, carvacrol, thymol, sustainable aquaculture, *Sparus aurata*, functional networks, interactome, mucosal-associated lymphoid tissue (MALT), oral immunization, fish nutrition, pathogen challenge

INTRODUCTION

INTRODUCTION

1. Aquaculture industry growth and challenges

1.1. Aquaculture global trends, challenges and sustainability

The global increasing demand for healthy and nutritious fish and seafood products, derived from global demographic growth, rising incomes and changing consumption patterns, is a challenge that depends in great part on the growth of the aquaculture production sector. The Food and Agriculture Organization of the United Nations (FAO) has published the latest statistical information on global aquatic production, both from aquaculture and fisheries. In 2019, the global aquaculture production was of 120.1 million tonnes, a 3.6% more than in the year before [1]. The aquatic production has been continuously growing during the last three decades at an average annual rate of 2.5%, even surpassing the world population growth rate of 1.6% [2]. According to the FAO's 2020 report, the global consumption of aquatic products per capita has increased from 9.0 kg in 1961 to 20.5 kg in 2018, growing approximately 1.5% per year. For the sixth consecutive year, aquaculture production in 2018 exceeded fisheries products in the market by 17.1 million tonnes. In the context of the projected increase of aquatic products consumption, aquaculture production will need to grow in parallel during the incoming decades, while fulfilling the 2030 Agenda Sustainable Development Goals (SDGs). To provide social and environmental benefits, the adoption of new and more sustainable fish production systems is advisable and required.

Nonetheless, these impressive numbers should not overshadow the considerable decline of the global annual growth rate of the aquaculture industry observed in the recent years. After decades of year-on-year growth rates of 6%-10%, 2018 has seen an increase of just 2.0% over 2017. In fact, the average annual growth rate of

aquaculture is projected to slow down from 4.6% in 2007–2018 to 2.3% in 2019–2030 [2].

The aquaculture rapid expansion increases its own vulnerability to many serious challenges that hamper the sector's development, in some cases undermining its ability to achieve the sustainable necessary outcomes, and affecting negatively consumers' opinion and the sector credibility [3]. A number of factors are expected to contribute to this slowdown, including the broader adoption and enforcement of environmental regulations, the decrease in the availability of water and suitable production locations, the decrease in productivity gains, the increasing outbreaks of aquatic animal diseases related to intensive production practices, and all the constrains associated to climate change [2].

Experts consider that both intensified production systems and climate change favor the occurrence of disease outbreaks due to the farming of more stressed and immuno-compromised animals, and the evolution and spread of more virulent pathogens. This qualifies aquatic animal diseases as one of the major limiting factors for aquaculture development [3; 4]. Previously unreported pathogens that cause new and unknown diseases are forecasted to emerge and spread rapidly, causing outbreaks and major production losses every three to five years [2]. Changes in temperature and weather events may negatively affect the water quality and may increase the transmission of infectious diseases as well as to contributing to their geographic distribution expansion [3; 4].

In order to prevent disease outbreaks, indiscriminate prophylactic use of antibiotics and chemicals associated to intensive aquaculture practices can still be observed among some of the major aquaculture producing countries [5; 6]. However, the recurrent use of such therapeutics has serious side-effects on the aquaculture system, not only by compromising animal's immunity, but also due to the potentiation of antibiotic-resistant bacteria [4]. In the European Union (Regulation 1831/2003/EC), the ban on antibiotics as growth promoters in animal feeds due to the antibiotic resistance threats have forced the animal production industry to adopt

antibiotic-free productions [4; 7]. This has been coupled with the development of more sustainable alternative and/or complementary strategies to reduce the use of chemotherapeutic drugs in aquaculture [8].

In addition, the latest report on Earth and Climate Change from the Intergovernmental Panel on Climate Change (IPCC) has underlined that it will be impossible to keep global temperatures at safe levels unless there is a transformation in the way the world produces food and manages the land [9]. Under this scenario, the aquaculture sector has a responsibility in this road towards the improvement of its environmental and economic sustainability. Therefore, in order to successfully solve the great challenges that the aquaculture sector is facing, research and innovative initiatives must be directed towards optimizing its efficiency and productivity, both in small and large-scale systems, as driven forces supporting the sustainable growth of this industry. This concept has been gradually assimilated by all the actors of the aquaculture's chain of value, resulting in a continuous decrease in the aquaculture carbon footprint through reduced greenhouse gas emissions. The reduction in use of freshwater and land resources, the improvement of feeding management and development of effective ingredients and additives, the domestication of local species, and the innovation in farming practices are key integrative efforts to achieve the aimed sustainability and increased efficiency of the aquaculture industry [3; 10].

1.2. The aquaculture of gilthead seabream (*Sparus aurata*)

1.2.1. Relevance of the species

The gilthead seabream (*Sparus aurata*) (*Figure 1*) is commonly found throughout the Mediterranean Sea, although less frequent in the eastern and southeastern Mediterranean regions and very rare in the Black Sea. It is also present in the Atlantic Ocean from the British Isles to Cape Verde and around the Canary Islands.

It is a benthopelagic fish naturally found in coastal environments, inhabiting seagrass beds, rocky and sandy bottoms, as well as the surf zone until depths of about 30 m. Adults may be found up to 150 m deep. The gilthead seabream is an euryhaline species, commonly entering brackish waters where the regular salinity changes occur. It is a carnivorous fish, but accessorily herbivorous, that may be sedentary, solitary or form small aggregations. Regarding its reproductive biology, this species is a protandrous hermaphrodite that matures as functional males in the first two years (20–30 cm) and later turn into females (33–40 cm). Spawning naturally occurs from December to April, when water temperatures are 13–17ºC [11].

Figure 1. Gilthead seabream (*Sparus aurata*).

The gilthead seabream is a relevant species for aquaculture, representing one of the main farmed species in the Mediterranean area. It is mainly farmed intensively in sea cages, and occasionally in estuarine ponds in land in almost all Mediterranean countries. The main producer countries of gilthead seabream in Europe are Turkey with 85,000 tonnes (representing 33.7% of total production), Greece with 65,300 tonnes (25.9%), Egypt with 36,000 tonnes (14.3%), Tunisia with 16,000 tonnes (6.3%), and Spain with 13,521 tonnes (5.4%). Its cultivation is also carried out in Italy, Cyprus and Croatia, and there are smaller productions in Malta, Israel, France,

Portugal, Albania, Algeria, United Arab Emirates and Bosnia, among others (*Figure 2*). The culture period varies with location and water temperature, but usually takes between 18 and 24 months for a specimen to reach 400 g from hatched larvae. Commercial size can vary from 250 g to more than 2.0 kg depending on the target market and consumers' preferences [12].

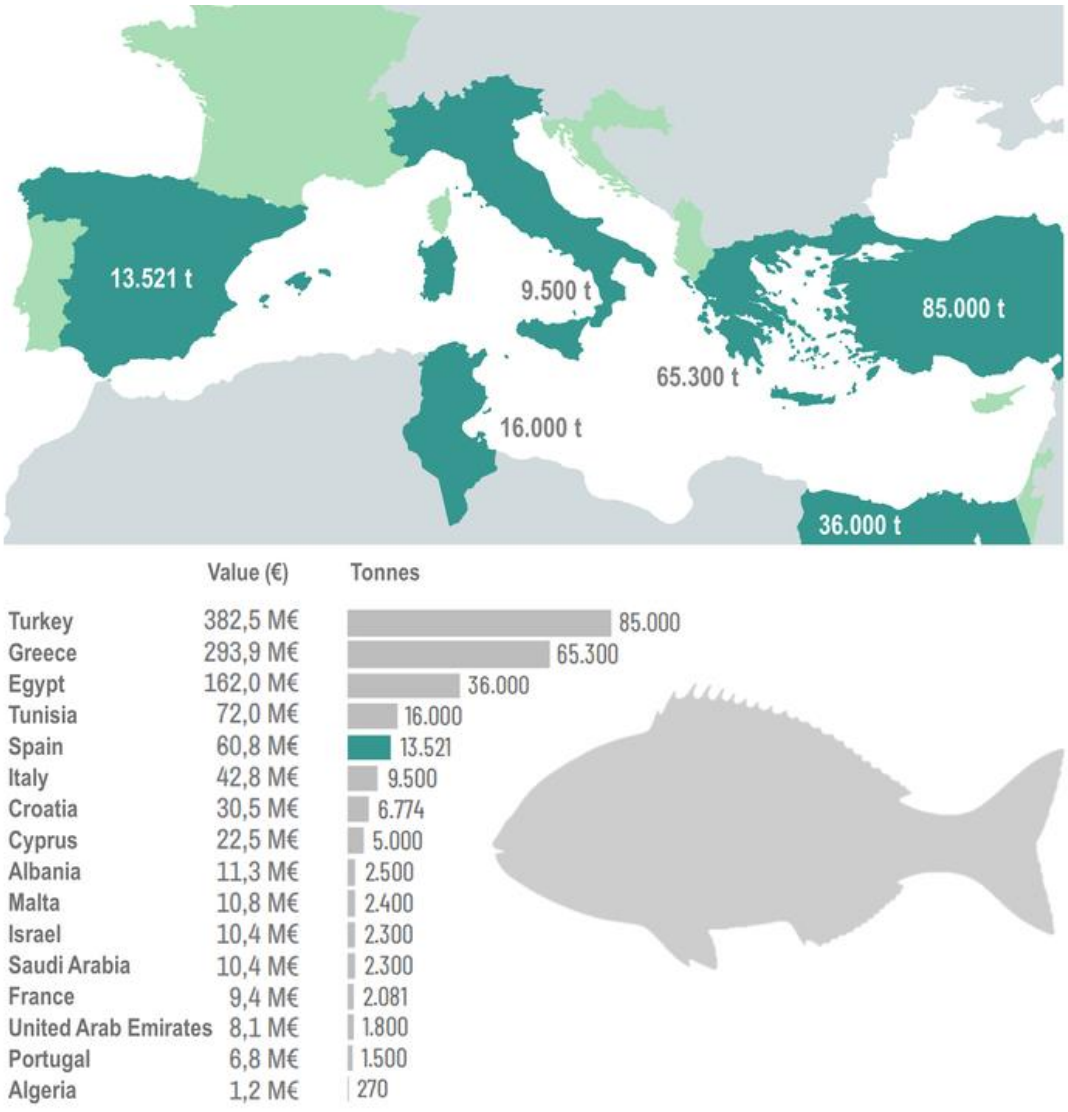


Figure 2. Distribution of aquaculture production for gilthead seabream (*Sparus aurata*) in the Mediterranean area in 2019 by volume (tonnes, indicated as "t" in the map) and values (millions of euros, detailed on bottom left). Adapted from FAO, 2020 [12].

The gilthead seabream is characterized by its high survival rate and flexible feeding habits, as well as its high adaptation to captive rearing, which has resulted into its domestication, attributes that make this species particularly suitable and attractive for aquaculture. However, a considerable decrease in the harvest of aquaculture seabream was estimated for 2020, mainly associated to the effects of the occurrence of punctual extreme weather phenomena and losses caused by pathologies [12].

1.2.2. Predominant diseases affecting farmed gilthead seabream

The most frequently reported disease that affects the production on-growing stage of gilthead seabream in the all the Mediterranean area is caused by the monogenean gill ectoparasite, *Sparicotyle chrysophrii* (*Figures 3, 4* and *5*), causing mortalities up to 30% and with the highest prevalence occurring in late spring and summer. The "Winter Syndrome" or "Winter Disease" is another frequently reported disease at this production stage, frequently affecting gilthead seabream during the "cold" period of January to May [13]. Furthermore, *Pseudomonas anguilliseptica* is one of the main agents responsible for outbreaks associated with the "Winter Disease", being considered a more opportunistic pathogen whose infections usually occur when fish are under environmental stress [14]. Regarding the hatchery phase, bacterial infections mainly associated to *Vibrio* spp., and betanodavirus infection are the most common affecting seabream larvae, fry and juveniles. These data were obtained from a recent survey in which a total of 50 production units (31 with on-growing, 16 with hatchery or nursery and three with processing plants) from 27 companies, located in 10 Mediterranean countries (Croatia, Cyprus, Egypt, France, Greece, Italy, Portugal, Spain, Tunisia and Turkey) have participated [13].

Figure 3. Adult *Sparicotyle chrysophrii* from the gills of gilthead seabream (*Sparus aurata*) © Ivona Mladineo

Experts have ranked the sparicotylosis as the most important disease affecting specifically gilthead seabream. The infection by *S. chrysophrii* causes fish lethargy due to hypoxia and severe anaemia [15]. The reported gills' histopathological lesions of the infection include lamellar shortening, clubbing and synechiae, proliferation of the epithelial tissue with resulting fusion of the secondary lamellae, and marked presence of chloride cells [16]. In addition, the spleen of parasitized specimens is generally increased in size and in the number of splenic melanomacrophage centres, suggesting increased erythrocyte destruction, and tissue catabolism and degeneration [17]. Secondary infections with other parasites and bacteria are also commonly found in fish infected with *S. chrysophrii* [18]. Consequently, sparicotylosis may reduce growth rate, increase the total feed conversion rate (FCR), increasing the feed requirement for > 50,000 tons in Mediterranean production [19]. The disease also makes the fish more vulnerable to handling and environmental stressors, and potentially causing mortalities reported within statistical records in the category "other causes".

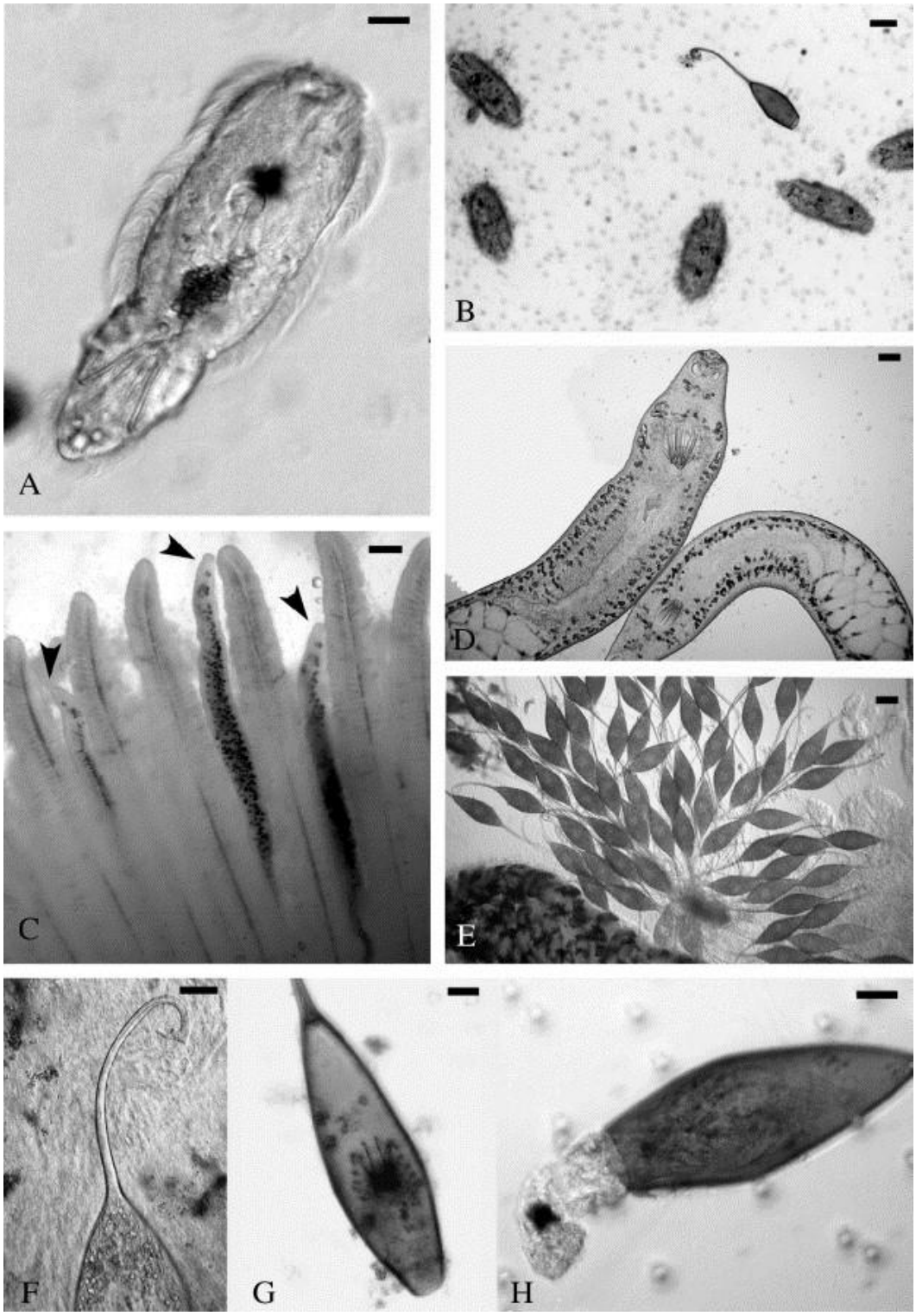


Figure 4. Microscope observation of different *Sparicotyle chrysophrii* developmental stages. A) Freshly hatched oncomiracidium larvae of *S. chrysophrii*. B) Immobilized oncomiracidia after formalin treatment. C) Adults attached to the gills of infected gilthead seabream. D) *S. chrysophrii* adults isolated from the gills. E) Intermingled group of eggs. F) Detail of an egg showing the typical hooked posterior end. G) Egg treated with formalin with a dead larva inside. H) Egg treated with limoseptic with a dead larva stuck at the opening end [15].

In nature, most parasites are normally present in small number on their hosts. However, the high densities that characterise intensive farming drive the burden of parasites in the farming area and increase exposure to both farmed and susceptible wild fish hosts [20]. This is of special relevance in sea cages where nets can represent an optimal reservoir for the parasite. Management of diseases comprises a significant part of operating costs in fish mariculture, and metazoan parasites are among the most problematic and challenging. Besides costs issues, parasites have become a significant animal welfare concern, while the chemical control methods usually employed for their treatment and control are under increased environmental scrutiny and government regulation [21; 22].

In the particular case of gilthead seabream, there are still no prophylactics against *S. chrysophrii* [23] and the only treatment available for the control of this parasite are on-site sea cage treatments with formalin baths and other chemicals used in routine disinfections [15]. Despite their effectivity, chemical concentrations required for controlling and treating parasites, particularly formalin, were observed to be noxious for several fish species, including gilthead seabream [24]. The toxicity of formalin leads to the disturbance and imbalance of several physiological functions, particularly affecting respiration and haematological processes, due to the histopathological damages in gills and haematopoietic organs [24]. In turn, parasites are prone to develop resistance to frequently applied chemical treatments. A clear example of this situation, that may be extrapolated to the Mediterranean region, may be the Atlantic salmon farming industry, which is currently struggling to control salmon lice, one of the major obstacles for the further growth of the industry [25].

These factors coupled to the environmental issues associated with chemical treatments in the open sea and the apprehension about human health risks [26] force the development of alternative procedures for promoting sustainable and environmentally friendly aquaculture practices [27]. In addition, taking advantage on the intrinsic host behaviour and immune response against parasites represents an interesting approach for disease management, aiming a shift in the current

disease control paradigm from reactive-based post-infection control to pre-infection prevention methods [22].

Figure 5. *Sparycotyle* spp. parasitizing on the gills of gilthead seabream (*Sparus aurata*) [28].

2. Functional feed additives as sustainable prophylactics for aquaculture

The traditional use of chemotherapeutants in aquaculture is decreasing and evolving to more sustainable alternatives due to the emergence of resistant pathogenic strains, their environmental impact, and the accumulation of trace residues in fish [29; 30; 31; 32]. These concerns have encouraged the development of reliable, safe and environmentally friendly methods to prevent disease, such as vaccines and functional feeds. Since disease outbreaks are intimately related to the animal's physiological and immunological status, the application of functional nutritional strategies, such as the use of immunostimulatory additives as sustainable prophylactic tools to improve fish health management in fish farms, has

gained considerable attention over the last decade [33; 34]. Besides benefiting fish growth and feeding efficiency, functional aquafeeds may promote fish immunity and welfare. These benefits are obtained from the inclusion of specific (micro-) ingredients and bioactive compounds that aim targeting specific functions or metabolic products that will enhance the fish immune capacity when challenged, and consequently reduce stress [8]. A large number of additives or feed supplements, such as probiotics, prebiotics, synbiotics, postbiotic immunostimulants, nucleotides, phytogenics, and vitamins and minerals, are currently available for their inclusion in functional feeds (*Figure 6*). A brief overview of their properties and function on the organism of aquatic species is described as follows.

Figure 6. In the design of functional feeds, a wide range of feed additives can be used to improve fish performance beyond the species nutritional requirements to improve growth and feed utilization. These functional feeds are specially designed to support the health and stress resistance of aquatic farmed species to the culture related practices. Probiotics, prebiotics, synbiotics, postbiotic immunostimulants, nucleotides, phytogenics, and vitamins and minerals, are among the currently available functional feed additives for their inclusion in aquafeeds.

2.1. Probiotics

In aquaculture, probiotics are live, dead or a component of microorganisms which are administered orally through diet or to the rearing water, conferring health benefits to host by improving disease resistance, health status, growth performance, feed utilization, stress response or general vigor. Those health benefits are achieved at least in part by the modification of the host and environment microbiota communities [35]. The probiotics category may include different bacteria, bacteriophages, microalgae and yeast, which have been widely used in aquaculture as mono or multi-strain solutions via feed supplements or water applications. Commonly used probiotics in aquaculture include members of the *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Enterococcus*, *Carnobacterium*, *Shewanella*, *Bacillus*, *Aeromonas*, *Vibrio*, *Enterobacter*, *Pseudomonas*, *Clostridium*, and *Saccharomyces* genera. Multispecies probiotics may be more effective than single-strain probiotics as different strains present in multispecies probiotics increase the chance of their survival in the gut and may exert a stronger synergic effect on the host [36].

Health benefits promoted by probiotics in aquaculture has been extensively reviewed [37; 38; 39]. The ability of probiotics to promote and/or improve health is related to their capacity to stimulate the host's immune response and to inhibit the growth of pathogenic bacteria. Probiotics can interact with or antagonise other enteric bacteria by resisting colonisation or by directly inhibiting and reducing the incidence of opportunistic pathogens. Competitive exclusion has been suggested as being the primary mode of action of probiotics. By means of competition for attachment sites and nutrients, such as iron, the probiotic strains occupation of the gastrointestinal mucosal epithelium and mucus prevents further pathogen's colonization [37]. Probiotics may exert antibacterial effects through the production of molecules with bactericidal activity, such as bacteriocins, siderophores, lysozymes, proteases and/or hydrogen peroxide. Besides, the alteration of the intestinal pH due to the generation of organic acids may also inhibit the growth of pathogenic bacteria [8].

Probiotics can also enhance health via host's physiological or immune modulation [35; 40]. It has been reviewed that probiotics can affect elements of the non-specific immune system, such as increasing leukocytes following treatment. In addition, probiotics enhance growth performance and feed utilization in aquatic animals through the promotion of digestive enzymes activity, such as alginate lyases, amylases, and proteases [37]. Probiotics also provide a more favourable environment for fish through the reduction in the proliferation of pathogenic bacteria and harmful phytoplankton in the rearing water, as well as through the bioremediation of organic wastes [8].

Commercial probiotics have been used in aquaculture since the early 1980s, which are mostly from terrestrial sources and not from the environment in which the aquatic animals live or from the host itself. Recent studies have focused on using host-related microbiota as a probiotics source since they are naturally established within the host defence system and revealed further beneficial effects [41].

2.2. Prebiotics

Prebiotics are non-digestible carbohydrates that have beneficially impacts on the fish intestinal microbiota growth and/ or activity, and the fish immunity; thus, improving host's health. The immunomodulatory activity of prebiotics is mediated through direct interactions with the host's innate immune system or by modulating the growth of intestinal commensal microbiota, as they are used as energy sources for the enteric bacteria. To be considered as prebiotics, these functional ingredients must be resistant to digestion, fermentable by beneficial intestinal bacteria and capable to promote the growth and/or activity of microbiota groups linked with health and welfare of the host. Several prebiotic products are widely applied in aquaculture, such as inulin, fructooligosaccharide (FOS), mannan oligosaccharide (MOS), galactooligosaccharide (GOS), arabinoxylan-oligosaccharide (AXOS), and isomaltooligosaccharide (IMO) [38].

Contrarily to probiotics, prebiotics are not microorganisms, but rather indigestible dietary ingredients that benefits the commensal enteric microorganisms. They can be naturally found in numerous plants, in particular the ones rich in fructan, as for instance, onion, garlic, artichoke, kiwi and soybeans, besides the oats and wheat, and plants that are rich in inulin, such as jicama and chicory root [42]. Prebiotics favor the escalation of probiotics in the gut due to their ability to digest them, while pathogens lack necessary sacchrolytic enzymes [43].

Besides benefiting the gut microbial population, these functional molecules also affect the bacteria responsible for the production of intermediate metabolites. These compounds, the short chain fatty acids (SCFAs), are well-known for their anti-inflammatory properties, assisting in the regulation of the immune system. They also directly enhance the gut epithelial barrier function and its phagocytic capacity. For that reason, they are also called as immunosaccharides. Prebiotics modulate the immune response also by interfering with bacterial-epithelial attachment and thus, preventing the adhesion of pathogens [43].

2.3. Synbiotics

Synbiotics consist of a combined application of prebiotics and probiotics. The synbiotic concept involves conferring the benefit of both probiotics and prebiotics to aquatic animals, mainly due to their synergistic effect. Their combined use aims to improve the survival of the probiotic organism and its fermentation efficiency, since the required specific substrate is readily available when administered together. Hence, the simultaneous presence of probiotics and prebiotics magnifies their beneficial outcomes upon the host, providing more benefits for aquatic animals when compared with the single addition of probiotics or prebiotics [34; 38].

Synbiotics have been used since 2005 in aquaculture to promote growth and immunity of aquatic animals. As part of a synbiotic, the prebiotic (*i.e*, FOS, MOS, GOS, AXOS, IMO or inulin) is hydrolyzed to mono- or disaccharides, selectively

increasing the probiotic biomass and colonization that is established by specific crosstalk between bacteria and the intestinal epithelial cells of the host, ultimately improving host welfare and performance [44].

2.4. Postbiotics

Postbiotics include any substance released by or produced through the metabolic activity of probiotic microorganisms that exert direct or indirect beneficial effects upon the host. Heat-killed probiotics also function as postbiotics as they retain important bacterial structures with potential to exert biologic activity in the host [45]. As postbiotics do not contain live microorganisms, the risks associated with their intake are minimized [39].

During their growth, microorganisms produce biopolymers with different chemical properties. that can be released outside the bacterial cell wall, forming a heterogeneous group of substances called exopolysaccharides (EPSs). Among the vast list of compounds produced by microorganisms and reputed as postbiotics [46], a popular example of EPSs are the β-glucans. β-glucans are complex polysaccharides found in yeasts, mushrooms, algae or cereals, such oats and barley, that are often reported for their immunomodulatory properties, being used as part of both prophylactic and therapeutic strategies. There is structural variability between β-glucan molecules that influences their chemical properties and functional activity. Immunomodulation effects are usually associated to β-glucans with higher molecular weight, although the direct relationship between structure and activity remains unclear [47]. The principal reported benefits of β-glucans in fish are immune stimulation and modulation, improved resistance to stress, and increased growth and survival. Anyway, those results may vary among studies depending on glucan source, dose, fish species and age [48; 49].

2.5. Organic acids

Organic acids, such as citric, fumaric, formic, lactic, butyric and propionic acids, have traditionally been used as storage preservatives in food and feed ingredients for preventing product deterioration caused by fungi and microorganisms [50]. Currently, they are widely applied in farmed animal feeds to control bacterial pathogens, although their effects on growth, nutrient utilization, mineral availability and gut microbiota are also documented [51]. These acids are commonly referred to as SCFAs and are produced through the microbial fermentation of carbohydrates by various bacterial species under different metabolic pathways and conditions. Some lower molecular weight organic acids are also naturally formed within the large intestine by anaerobic microbiota communities, although most organic acids commercially used in feeds are produced synthetically. Blends of organic acids are particularly applied in animal feeds as they present a broad spectrum of antimicrobial activity against a wider range of potential pathogenic bacteria, with potential synergistic effects upon growth performance and nutrient utilization. Organic acid-supplemented diets have consistently been reported to show improved feed intake, growth, feed utilization efficiency, overall health and disease resistance in farmed animals, including aquaculture relevant species. They were also observed to reduce the negative impact of alternative plant-based proteins increasingly used in aquafeeds [52]. Moreover, as a result of diet acidification, the reduction in P and N excretion due to improved mineral utilization favors the formulation of more environmentally friendly aquafeeds. The reduction in the microbial load of the excreted faecal matter from farmed fish fed organic acids-supplemented feeds might benefit water culture systems [51].

2.6. Nucleotides

Nucleotides (NT) are low-molecular weight intracellular compounds that represent the building blocks of nucleic acids, playing key roles in nearly all biochemical processes. Under healthy conditions, NT are produced of through the recycling of

dead cells and the degradation of RNA and DNA (salvage pathway); or through direct *de novo* synthesis from amino acids precursors, such as glutamine, formate, glycine and aspartic acid. However, due to their high energy requirement, synthesis of NT may become limiting under stressful conditions, such as infection or during fast growth and developmental periods [53].

Besides their possible involvement in diet palatability, fish feeding behavior and biosynthetic pathways, dietary supplementation of NT have shown promising results in enhancing immunity and disease resistance in aquaculture relevant species [54]. Research on dietary NT has shown that they may improve aquatic species growth in early stages of development, enhance larval quality via broodstock fortification, improve intestinal morphology, increase stress tolerance, as well as modulate the innate and adaptive immune responses, showing enhanced resistance to viral, bacterial and parasitic infections. In addition, NT were also found to reduce the negative effects of alternative plant-based proteins which leads to the increase of the feed efficiency, increasing growth and health performances of aquaculture species. Thus, it is hypothesized that NT may be beneficial and essential nutrients in plant protein-based diets, particularly for young fast-growing fish [53].

2.7. Vitamins and minerals

Vitamins cannot be synthesized by animals in sufficient amounts to meet their nutritional and physiological needs and, therefore, must be obtained from the diet. Vitamins play essential roles in the maintenance of normal metabolic functions, acting principally as cofactors for enzymes, whose inadequate supply leads to reduced enzyme activities resulting into poor growth, low survival and increased susceptibility to infections, among other deficiency signs and symptoms [55]. For instance, many fish and crustacean have limited ability to synthesize vitamin C due to the absence of L-gulonolactone oxidase that is responsible for its biosynthesis. Thus, signs of deficiency are observed when vitamin C is excluded from the diet.

Vitamin C has been known as a major antioxidant and immunomodulator micronutrient, correlating with enhanced aquatic animal performances. Vitamin C is as a water-soluble antioxidant able to scavenge free radicals including ROS and reactive nitrogen species, avoiding cellular damages from radical components. It has also been proposed to increase fish immune response, promoting serum bactericidal activity, phagocytic activity, immunoglobulins levels and lysozyme activity [56].

Trace minerals, such as Zn, Mn, Cu and Se, are required in small quantities, but participate in a wide variety of biochemical physiological processes. General function of minerals includes being components of key enzymes and vitamins, structural constituents of tissues, balance of osmotic pressure, and transmission of nerve impulse and muscle contractions. Those referred, have been particularly associated with an improvement of the immunity or function that support immunity, and antioxidative protection in cultured aquatic animals [56].

2.8. Phytogenics

Phytogenic feed additives, sometimes also referred to as botanicals or phytobiotics, are a group of natural substances of plant origin, derived from herbs, spices and plant whole parts or extracts. These substances are used as feed additives in animal nutrition and have been gaining increasing interest in the aquaculture sector during the last decade [57]. Among other additives such as the ones previously mentioned, phytogenics are important prophylactic and therapeutic tools that exert a positive impact on the health and welfare of farmed animals, as well as production systems, without any known environmental and hazardous problems associated to their administration. In this context, phytogenics are viewed as promising sustainable solutions for conventional and antibiotic-free animal nutrition [4; 57].

The health-promoting properties of phytogenics have been extensively reviewed in different aquaculture species [57; 58; 59; 60; 61; 62; 63]. Several studies reported

numerous phytogenics to enhance the immune response of fish, in which several classical immune markers, such as lysozyme, complement, antiprotease, meloperoxidase, reactive oxygen species (ROS), reactive nitrogen species, phagocytosis, respiratory burst activity, nitric oxide, total haemocytes, glutathione peroxidase (GPx), phenoloxidase and antibacterial activity in both serum and mucus samples were reported to be positively affected by the administration of phytogenics in aquafeeds [8; 57]. Phytogenics were also reported to effectively enhanced fish growth and disease survival, regardless of the trophic level of the fish species studied, the duration of the treatment or the type of material used [57].

Plant secondary metabolites have functional roles independent from plant growth and development; thus, protecting plants from herbivore and pests, or acting as chemoattractants for pollinators [64]. These bioactive compounds are broadly found in aromatic plants extracts, such as essential oils (EO), and are usually present as mixtures, mainly represented by phenolics and terpenes that are chemically characterized by their aromatic rings [65]. Therefore, their benefits as dietary supplements are subject to the variability and complexity of the aromatic compounds' mixture, apart from their synergistic effect, their origin, the dietary inclusion level and their pharmokinetics [66]. These compounds are used for their recognized growth promoting, antimicrobial, immunostimulant, antioxidant, anti-inflammatory and sedative properties. In particular, phytogenics derived from Lamiaceae family and *Allium* sp. are among the most widespread administrated plant-based additives in aquaculture [60] and livestock [67; 68]. Although they can be found worldwide, some representatives of this group of aromatic plants (i.e., oregano, thyme, basil, menthe, rosemary, sage, marjoram, garlic and onion, among others) are particularly present and traditionally consumed in the Mediterranean area and appreciated in terms of human nutrition and therapy [69; 70]. Combinations of different phytogenics are also promising strategies for the formulation of functional feeds duet to their potential synergistic effects. Under the vast range of phytobiotics that have been tested in aquafeeds, this thesis is focused on the evaluation of a blend of garlic essential oil and the predominant bioactive

compounds found in oregano and thyme – carvacrol and thymol –as a functional feed additive for aquaculture.

2.8.1. Garlic

Garlic (*Allium sativum*) and its organosulfur bioactive compounds are recognized for their therapeutical potential since ancient times. They have shown health-promoting and disease-preventing effects on several human common diseases, such as cancer, cardiovascular and metabolic disorders, blood pressure and diabetes; effects that have been demonstrated in several *in vitro*, *in vivo* and clinical studies [71]. Due to those health-promoting properties, these phytogenics have been also widely used as a feed supplement for farmed animals [72]. In aquaculture, the effectiveness of garlic extract as an immunostimulant, antimicrobial and antiparasitic agent has been demonstrated in several fish species [73; 74; 75; 76; 77]. Garlic-based treatments have been demonstrated to be particularly effective as a therapeutic agent against parasites [77; 78; 79], including monogeneans [80; 81]. Ajoene, one of the main garlic's organosulfurs, was described to interfere with parasite and host cell membrane protein and lipid trafficking, with irreversible detrimental consequences for the parasite [82].

Garlic dietary administration was also reported to positively impact the gut health of aquatic species through its ability to modulate the intestinal microbiota [83]. In fact, garlic is known for its wide-spectrum of antimicrobial activity that is attributed to the organosulfur compounds ability to penetrate the bacterial cell membranes, cause changes in the structure of thiol (-SH) containing enzymes and proteins, and lower the expression of important genes involved in the QS in bacteria. This effects consequently inhibit the growth of both Gram-positive and Gram-negative bacteria [84]. Many reports have described garlic bactericidal activity against common fish pathogenic bacteria [61]. These antibacterial properties can be also translated to the

skin mucus of fish fed garlic-supplemented diets, in which an increase of the skin mucus antibacterial capacity is observed [85].

Additionally, dietary garlic has been described to increase fish immune cells number and enhance their phagocytic capacity [73; 86; 87] Garlic organosulfur compounds have been also reported to stimulate inflammatory immune responses, promoting the release of pro-inflammatory cytokines, enhancing the proliferation of lymphocytes, macrophage phagocytosis and modulating the infiltration of immune cells [88]. It was also demonstrated that depending on the organosulfur compound considered, it can either augment cells' phagocytic function and, consequently, ROS production, or inhibit those cell's spontaneous ROS production [89]. This apparent antagonistic effect evidences the pleiotropic protective effects of garlic extracts and essential oils, being simultaneously capable of inducing immune responses and anti-inflammatory counteractions.

In accordance, garlic supplementation in aquafeeds has been also described to improve fish antioxidant status [90; 91]. The detoxification and chemoprotective benefits from various organosulfur compounds have been associated to their ability to scavenge free radicals and selectively enhance or suppress the synthesis of several antioxidant enzymes (at gene and protein level), such as cytochrome P450 enzymes or glutathione S-transferase (GST) [92], exerting a direct effect upon immune cells [93]. In this line, their anti-inflammatory activity upon immune and epithelial cells was associated to the inhibition of ROS production and the modulation of the NF-kB and MAPK signalling pathways [94; 95].

Besides, garlic inclusion in fish diets has been also reported to improve feeding efficiency and growth performance [83; 85; 96; 97], as well as fish flesh quality due to its hypolipidemic characteristics [61]. At physiological level, garlic reduces the levels of stress-related markers, potentially improving animal welfare [90; 98]. Overall, garlic's known therapeutical benefits, in addition to its economical and practical characteristics, suggest that the application of garlic-based phytogenics in

functional feeds may represent an effective prophylactic tool to be used in aquaculture health management.

2.8.2. Carvacrol and thymol

Oregano (*Origanum vulgare*) and thyme (*Thymus vulgaris*) are members of the family Lamiaceae native from the Mediterranean regions and Eurasia. They contain high amounts of essential oils and have been widely used in many countries as culinary herbs and for different medicinal purposes [99; 100]. They are among the most commonly used aromatic plants in animal feeds because of their richness in terpene phenolic compounds like carvacrol and thymol [58; 101]. These phytogenics and related bioactive compounds have been used in aquaculture for their wide range of properties such as antimicrobial, immunostimulant and anti-oxidative activities, and the ability to enhance intestinal absorption, to improve growth and even to reduce cumulative mortality [58; 101].

Carvacrol and thymol are particularly studied and recognized for their bactericidal activity, since their lipophilic character act as bacterial membrane permeabilizers with cytotoxic effects upon bacterial structure and function, leading to membrane expansion, fluidity and permeability, disturbance of the membrane-embedded proteins, respiration inhibition and alteration of ion transport. In addition, carvacrol and thymol were demonstrated to act as quorum sensing (QS) inhibitors, reducing bacterial biofilm formation. Carvacrol in particular, is able to inhibit bacteria motility, collapsing the proton-motive force, depleting the ATP pools and preventing the synthesis of flagellin [102]. This bactericidal property highlights the ability of these compounds to potentially modulate mucosal tissues associated microbiota. In fact, diets supplemented with phytogenics rich in these compounds have been reported to modulate the intestinal microbiota composition exerting beneficial effects upon fish gut health [103], as well as improving the skin mucus antibacterial capacity [104; 105].

Together with their well-studied bactericidal potential, these phenolic compounds are described to possess antioxidant, anti-inflammatory and consequent immunomodulatory properties [106]. The reported strong antioxidant activity of carvacrol and thymol rely on their ability to scavenge free radicals, inhibiting reactive oxygen species (ROS) and other oxygen radicals generated in cells and tissues [107]. Regarding their anti-inflammatory potential, carvacrol and thymol appear to interfere with the NF-kB and MAPK pathways, modulating the expression of pro-inflammatory and anti-inflammatory cytokines [108; 109]. In aquaculture, diets enriched with carvacrol, thymol or related phytogenics were described to improve overall fish antioxidant status [110; 111], as well as to significantly enhance leukocytes number and phagocytosis [110; 112; 113].

Regarding their antiparasitic potential, several bath treatments with different EOs rich in carvacrol and thymol proved to be effective anthelmintics [114; 115; 116]. The antiparasitic action of these compounds has been associated to their presence in the skin of fish after dietary supplementation [117].

3. Fish mucosal tissues as targets for functional feed additives

Aquatic farmed species are more dependent on mucosal barriers than their terrestrial counterparts, as they are continuously interacting with the aquatic environment and associated microbial community, which frequently contains a higher burden of antigens. Since mucosal barriers constitute the fish first line of defence against the surrounding environment and potential pathogens, an increasing trend to evaluate the impact of functional feed additives upon the mucosal immunity has been gained importance in recent years.

Regarding their vital immunological roles, fish mucosal tissues are characterized by a mucosa-associated lymphoid tissue (MALT), harbouring diverse myeloid and

lymphoid cells that are responsible for the host protection against pathogens and antigens, while tolerating beneficial microbiota colonization to maintain mucosa homeostasis [118; 119]. Six different MALTs have been described so far in teleosts. The gill-associated lymphoid tissue (GIALT), the gut-associated lymphoid tissue (GALT), the skin-associated lymphoid tissue (SALT), the nasopharynx-associated lymphoid tissue (NALT) and, the more recently characterized the buccal, and pharyngeal MALTs [120; 121]. Other mucosal immune systems have been hypothesized and are currently under study [122; 123] (*Figure 7*).

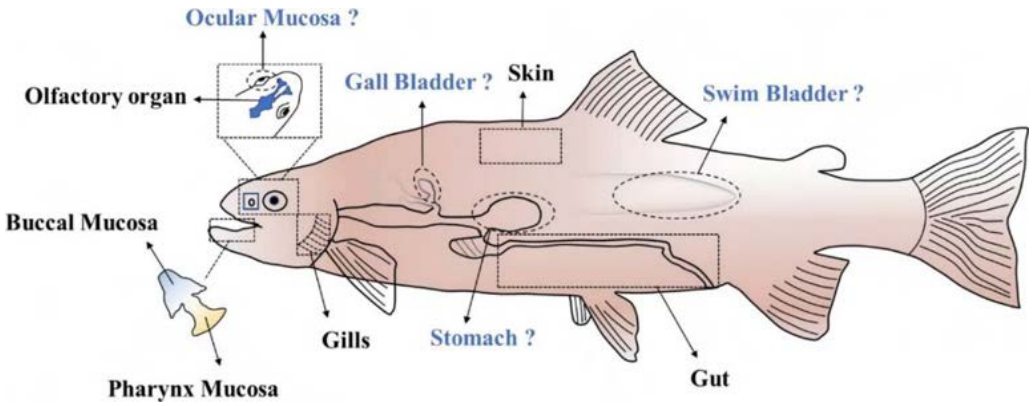


Figure 7: Studied and putative mucosa-associated lymphoid tissues (MALT) in teleost. "?" indicates potential fish MALTs that remain to be clearly characterized [123].

Despite the existence of other MALTs, the GIALT, GALT and SALT are the most studied and well characterized ones and therefore, they were selected as targets for the evaluation of the phytogenics'-based additive tested in this thesis. As follows, a small review of their correspondent mucosal tissues is presented, taking into account tissue functionality, basic morphological characteristics, role played in the defence of the organism, microbiota, and potential as targets for nutritional strategies.

3.1. Gills

The fish gills have several vital functions, as they are involved in the respiration, osmoregulation, nitrogenous waste excretion, pH regulation and hormone production. To ensure such exchanges between the organism and the outer external aquatic environment, a short distance between the blood and water, and a thin mucus layer covering gill's epithelia is necessary. Its surface also represents the largest organ-specific surface that interacts with the external environment [124]. Such semipermeable features characterized by large area and short distance promote a high efficiency of gas exchange, but also facilitate the uptake of foreign substances or invasion by infectious agents. In this sense, gills are a significant portal of entry for several pathogens. Gill diseases and/or damage cause substantial losses in the aquaculture industry, not only through an increased fish mortality, but also through the impairment of their growth, increase in FCR values, and associated sanitary and treatment costs [23; 125].

The external gill surface consists of apical plasma membranes and a glycocalyx attached to those membranes. Glycocalyx is a carbohydrate-rich layer that forms the outer coat of cells, composed by oligosaccharides, including sialic acid residues, present in glycolipids and glycoproteins, glycosaminoglycans and polysaccharides. Furthermore, the thin mucus at the gill surface consists mostly of gel-forming glycosylated glycoproteins called mucins secreted from mucous cells, which thickness must be balanced according to the short diffusion distances necessary for gas exchange. The continuous secretion of mucins by goblet cells leads to physical removal of attached pathogens or toxins, as well as to the establishment of commensal microbiota [126]. The content of immune molecules in gill mucus has not been examined to the same extent as for other mucosal tissues, such as the skin or the intestine. However, a long list of common molecules, such as lysozyme and immunoglobulins, indicates high similarities between mucus secretions produced by different mucosal tissues [124].

Fish gills, as other mucosal tissues, are particularly characterized by a MALT, the gill-associated lymphoid tissue (GIALT), harbouring diverse myeloid and lymphoid cells that are responsible for the host protection against pathogens and antigens, while tolerating beneficial microbiota colonization to maintain mucosa homeostasis [118; 119; 127].

Compared to other organs, gills are very high in MHC class I and II expression, which is predominantly associated to antigen-presenting cells including dendritic cells and macrophages. It has also been described for some fish species the presence of the interbranchial lymphoid tissue (ILT) mainly containing T cells embedded in the epithelium. In fact, increasing evidences points towards the gills as one of the major organs involved in the immune response [128].

For that reason, during the recent years gill immune responses were studied as a target for vaccines, by mean of bath immunization where the gill with its large surface is one of the promising sites of vaccine uptake [129]. Consequently, understanding gill physiology including uptake and immune response mechanisms is of utmost importance for designing effective therapeutics. The schematic comparative representation of the main immunological components of the GIALT in relation to other mucosal tissues in fish is depicted in *Figure 8*.

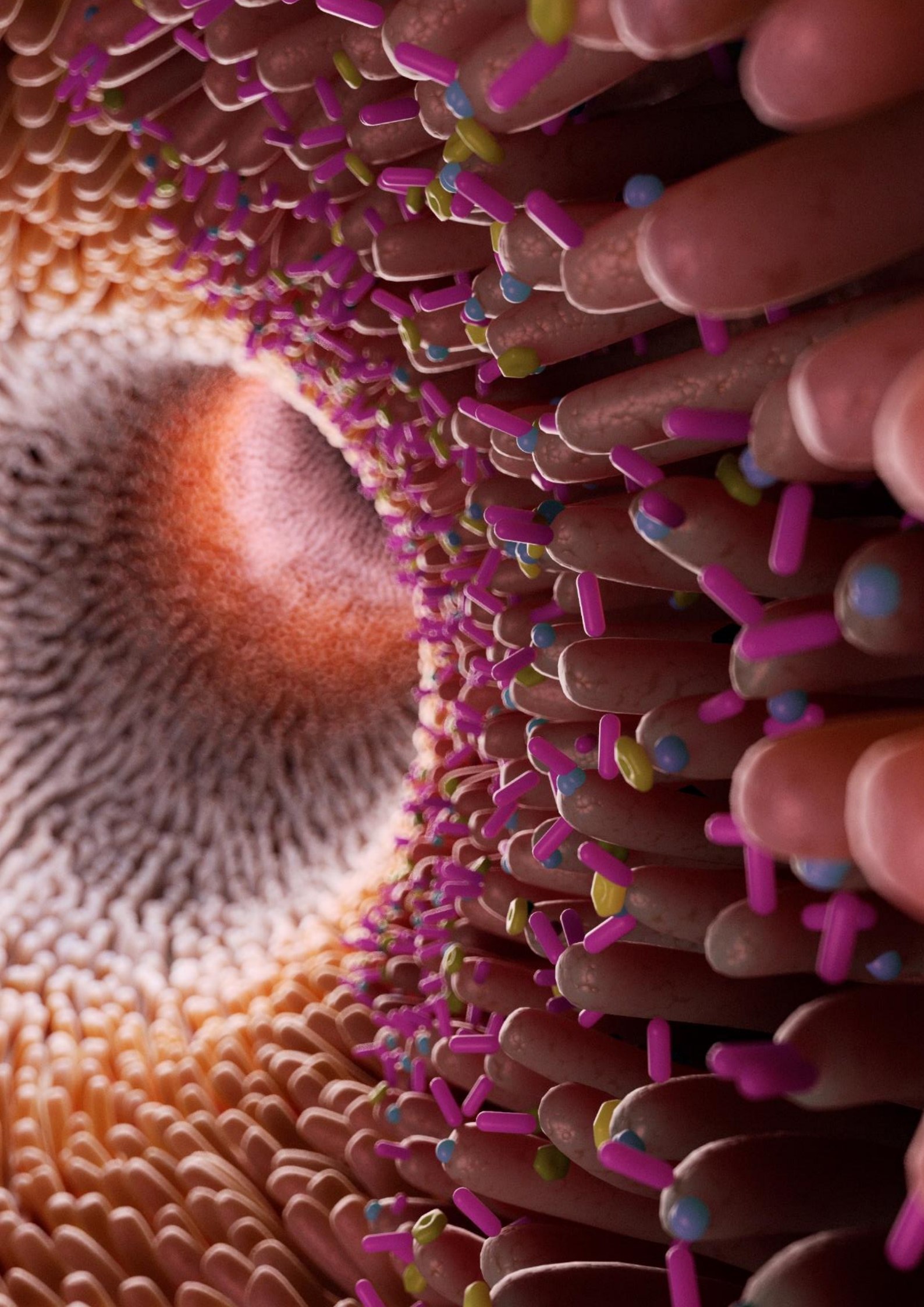
Gills support high populations of a wide range of bacterial genera. The cultivable microbial communities of the gills are typically of the same order or lower than those reported in the skin and gastrointestinal tract. In fact, it has been suggested that due to the continuous water current passing over the gills, this organ represents a tough habitat for microorganisms and that microbial colonization is more restricted to protected areas, such as clefts between pharyngeal arches and lamellae [124]. However, they represent a perfect environment for hematophagous ectoparasites, such as the *S. chrysophrii*, which despite the fish local and systemic response against its infection, may be able to modify the host's immunity in its own interest, representing a significant challenge for the health management of these parasites [23].

3.2. Intestine

The gastrointestinal tract of vertebrates is a multifunctional organ, which carries many important and diverse physiological functions, such as digestion, chyme lubrication, nutrient uptake, osmoregulation, nitrogen metabolism, appetite regulation and defence [130]. The intestinal epithelium of fish consists of a single layer of columnar epithelial cells, called enterocytes. The enterocyte apical membrane is characterized by the presence of microvilli, which form a brush border contributing to nearly 90% of the total intestinal surface area, depending on the area of the intestine and the fish species. The epithelial cells are also responsible for the enzymatic activity at the brush border membrane.

The gastrointestinal tract of fish is continuously challenged with food antigens as well as bacteria, viruses, parasites and toxins. Therefore, the gut epithelial cells are in continuous renewal, as well as being protected by a mucus layer that creates a physical and chemical barrier against external threats. Goblet cells are the dominant mucous cell type in fish intestine epithelium, which are responsible for mucus is discharge. As mentioned before, the main molecules found in the mucus are mucins, which play an important role in the maintenance of the epithelial barrier against pathogens. The gut mucus is also implicated in nutrient uptake and digestion, or even in the uptake of oxygen in the case of air-breathing fish. Thus, the intestinal mucus is a semipermeable barrier that allows the uptake of macromolecules required for nutrition, but acts in the same time as an effective barrier to particulate matter and microorganisms. Furthermore, this mucus layer acts as an important mechanism of innate defence that maintains mucosal tissue homeostasis [131]. Antimicrobial and other immune-related molecules are secreted into the teleost gut mucus, although proteases from luminal bacteria and their degradative ability against immune factors difficult to detect their presence in significant levels, being mostly observed inside fish intestinal goblet cells.

Among the several intestine's functions, defence is one of the most important, since the gut is one of the physical barriers that pathogen encounter and have to surpass



in order to invade the organism. Fish gastrointestinal tract contains a gut-associated lymphoid tissue (GALT) that has the remarkable capacity to differentiate between beneficial and potentially dangerous material, promoting protective immune responses against harmful microorganisms and toxins while accepting food antigens and commensal microbiota [132]. This MALT is of special relevance for the aquaculture industry, since farmed fish are generally fed commercial feeds, which therefore allows the manipulation of the fish health through functional feeds. In this sense, the schematic comparative representation of the main immunological components of the GALT in relation to other mucosal tissues in fish is illustrated in *Figure 8*.

Similar to higher vertebrates, fish gut is also characterized by neural and endocrine systems. Most activities involved in the physiological control of the gastrointestinal function during feeding or fasting periods are mediated by neuroendocrine systems, playing important roles in the overall regulation of digestion. Gastrointestinal hormones are synthetized and secreted by enteroendocrine cells distributed throughout the intestinal tract. This neuroendocrine system may also interact with the local immune system, in which changes in hormone secretion, such as cortisol for instance, might modulate the gut immune responses, the intestinal motility and the microbiome [133; 134].

Fish gut is characterized by a complex association of microorganisms that play critical roles in the health of the host at both local and systemic levels. This microbiota community consists of members of the Archaea and Bacteria, viruses, protozoa and yeasts. These microorganisms can be classified as allochthonous or autochthonous, depending on their location and residence within the gastrointestinal tract. While the allochthonous microbiota are mainly transient, passing through the lumen with food and digesta, the autochthonous microbiota comprises resident communities that live in the host mucus layer or in close proximity to the mucosae. Beyond their described nutritional functions, the gut microbiota is also recognized as a key component of the mucosal barrier function, competing against pathogens and participating in the host mucosal development

and immune response [135]. However, most of the studies available have focused on pathogens and disease, with limited information on the crosstalk between microbiota and the fish mucosae. Although the gut microbiomes of fish are well studied in comparison to other mucosal tissues, they remain partly described and their influence on the host is still poorly understood [136].

Moreover, the fish gut microbiota can be manipulated through feed and water. Altogether, despite its intricate immune system, the intestinal tissue became an attractive target for functional feeds in aquaculture. The rules that govern mucosal immunity, however, are very different from those that govern systemic immunity. Thus, implementing effective mucosal immunotherapies for aquaculture species requires an in-depth understanding of the mucosal immune system of fish.

3.3. Skin

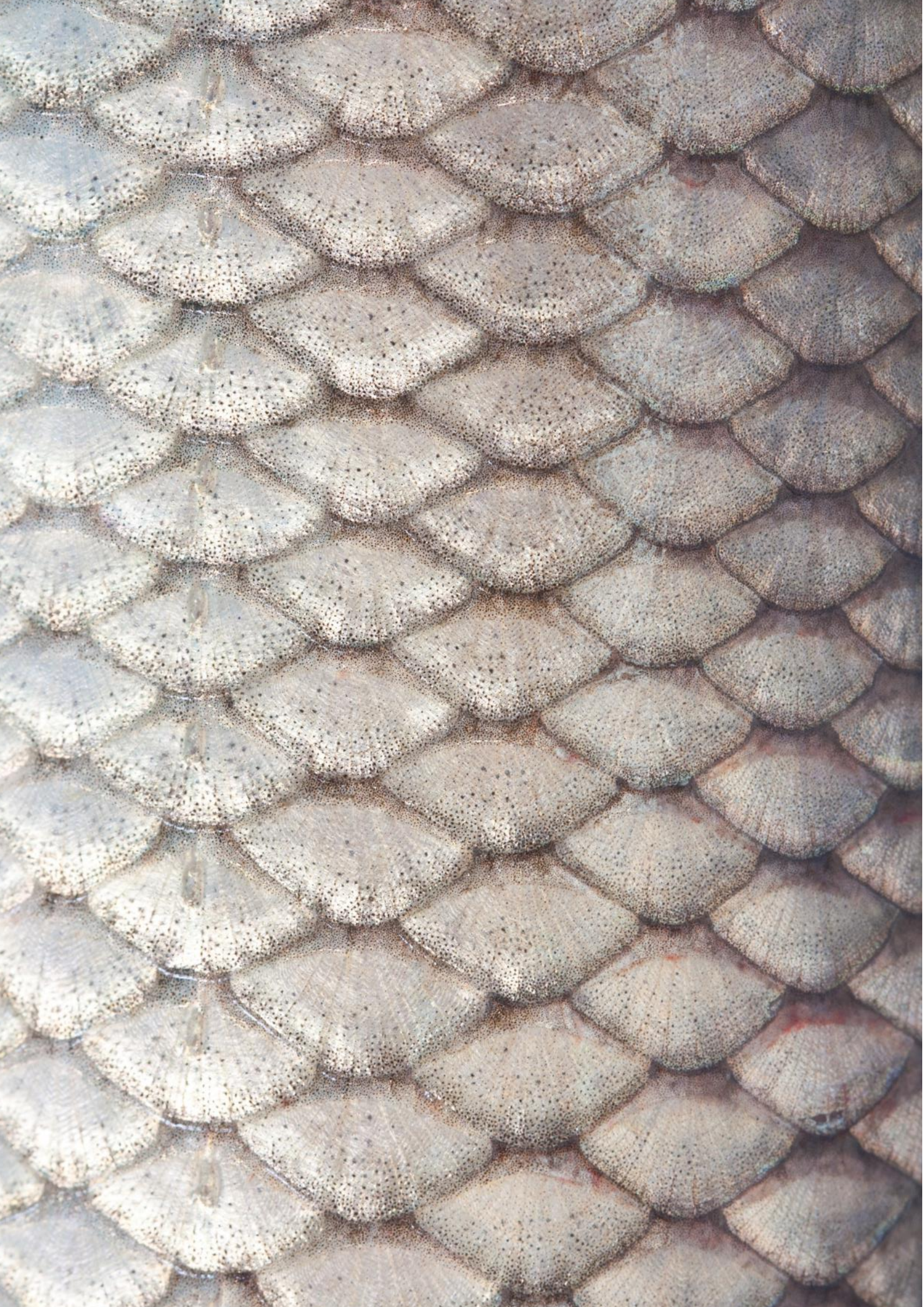
The structure and function of fish skin reflects the adaptation of the organism to the physical, chemical and biological properties of the aquatic environment. The fish skin covers the outer surface of the fish, including the body and fins, separating the organism from its environment and representing a critical interface for contact and communication with its external milieu [137].

The skin, sometimes referred to as integument, is the largest organ of the fish body. It has a complex structure that serves numerous vital functions, such as protecting fish from abrasion and predation, protecting from microorganism invasion and chemical insults, preserving hydrodynamics, maintaining osmotic balance, participating in gas exchanges, or communicating with the environment and other organisms through tactile, thermal and chemical sensors. In general, teleosts' skin is divided in three layers: the cuticle or mucous layer, the epidermis and the dermis. Contrary to mammals, the fish epidermis is a stratified epithelium of variable thickness which epithelial cells are majorly alive and metabolically active, retaining their capacity for mitotic division and being continuously replaced by living cells beneath. The skin epithelial cells are also involved in an exclusive wound repair

mechanism in which, after damage, these cells migrate collectively toward the wound, rapidly covering it and providing a protective barrier against opportunistic pathogens. In its turn, the dermis is mainly composed of dense connective tissue with a large amount of collagen fibers and typically containing bony scales, while the hypodermis consists of both loosely organized collagen fibers and a rich supply of blood vessels, which innermost layer contacts with the striated muscle underneath the skin. Skin is also characterized by its important mucosal immunity associated to the skin-associated lymphoid tissue (SALT), containing a variety of leukocytes, including lymphocytes, plasma cells, macrophages and granulocytes, some with the ability to migrate through mucous secretions [137]. Similar to the other mucosal tissues studied in this thesis, the schematic comparative representation of the main immunological components of the SALT in relation to the other mucosal tissues in fish is illustrated in *Figure 8*.

As being the largest fish mucosal tissue, the presence of the external mucus that covers all the living epithelial cells is one of its most distinctive features. This thin semipermeable mucosal layer separates the fish from its aquatic environment, trapping and immobilizing foreign particles and potential pathogens before they can contact with epithelial cells, being then removed from the skin mucosae by mucus constant secretion and replacement [138]. This dynamic characteristic of the fish mucus affects both quantity and quality of the substances present in it, representing one of the most important mechanisms of the skin innate immunity system [139]. As for the other mucosal tissues, this mucous layer is mainly composed by mucins and it is secreted by goblet cells, which are located in the epidermis. Additionally, the exterior surface of outermost fish skin epithelial cells is characterized by microridges that help retaining the mucus secretions on the skin surface [140].

The antimicrobial properties of epidermal mucus against pathogens, both bacteria and viruses, have been demonstrated in several fish species [141]. It is known that the repertoire of innate immune molecules contained in teleosts' mucus, such as



lysozyme, complement, proteases, immunoglobulins, lectins, among others, is more diverse than that of mammals. In particular, teleost skin is a major source of antimicrobial peptides (AMPs), such as hipposin, piscidin, pleurocidin, parasin, defensin and hepcidin, whose overall expression is of approximately 70% in the skin, while only 52% and 29% in the gills and gut, respectively [131].

Nevertheless, the fish epidermal mucus may also be an adhesion site for microorganisms that are adapted to evade or resist its immunological components. Epidermal mucus components can even be metabolized by some organisms in a mutualistic relationship between the fish and the skin microbiota. In fact, part of the multifunctionality of the epidermal mucus is derived from its microbiota content, which in turn can also interfere with pathogen colonization through antagonistic activity and competition for adhesion sites and/or nutrients [142].

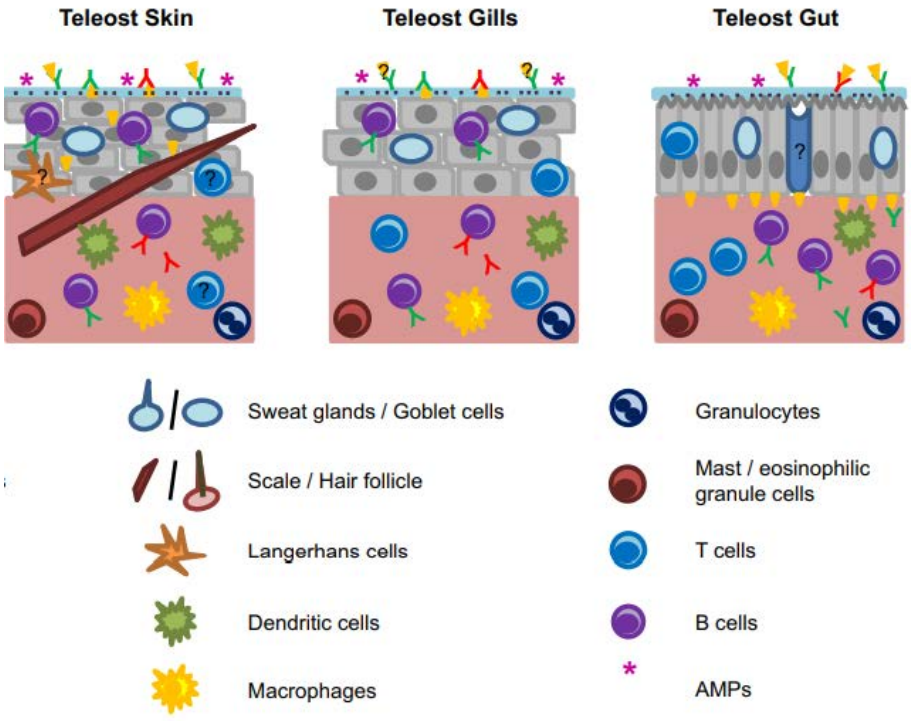


Figure 8. Schematic representation of the similarities and differences between teleost fish skin, gills and gut (adapted from Gomez et al. [131]).

4. Tools for evaluating functional feed additives

While numerous *in vivo* studies have demonstrated an improvement of the fish immune responses after the administration of functional feeds, the current information about the inherent effects of feed additives upon immunity is limited under *in vitro* conditions. In fact, most of the studies dealing with functional feed additives, particularly phytogenics, are exclusively focused in physiological or biochemical responses, and experimentally-controlled disease challenges, with few of them trying to elucidate the cellular and molecular mechanisms underlying their immunostimulatory capacity (Firmino et al. 2021 under revision, Chapter IV).

However, an array of new cellular and molecular tools has become available over the last several years, which allows deeper and wider understanding of those mechanisms. These data combined with classical approaches, like histological studies, humoral immune parameters, and biotic or abiotic challenges, can provide useful information for proper understanding of the mode of action of functional feed additives, an approach that contextualize organ and tissue responses under the cellular and gene expression levels. In this sense, omics tools can provide meaningful answers to characterize the complex teleost immune responses to the administration of phytogenics, in particular those regarding the less known fish mucosal immunity, in which intricate interactions occur between immune, epithelial, neuroendocrine, and mucus secreting cells, antigens and microbiota, and environmental signalling [143]. Modern omics technologies include genomics, proteomics and transcriptomics. Transcriptomic studies in aquaculture have gradually progressed from the traditional approach of single gene expression analysis to more recent high throughput sequencing techniques, including microarrays and transcriptome sequencing (RNA-seq) (*Figure 9*) [144].

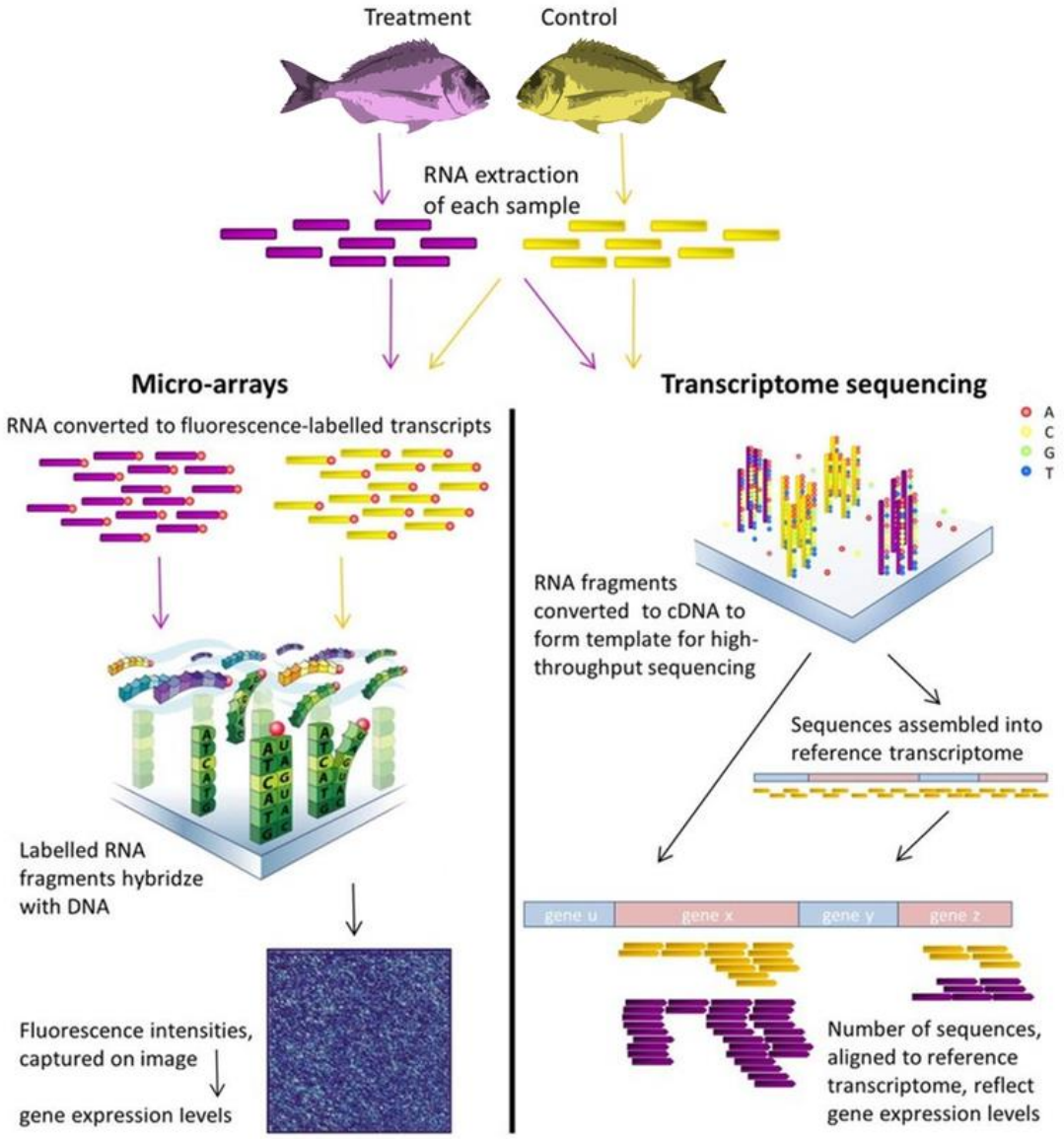


Figure 9: Schematic overview of transcriptomics approaches, using microarrays or transcriptome sequencing. Although the technologies differ, both approaches compare all the mRNAs present in biological samples under different conditions, and provide quantifications of the abundance of all gene transcripts for each sample (adapted from Wertheim, 2012 [145]).

Nutrigenomics in particular, have not only enhanced the understanding of biological markers for nutrition-related diseases, but they have improved the development of feed additives targeting the immunity in aquatic animals [144]. This useful approach allows to get a comprehensive understanding of different humoral

and cellular immune components present in fish, as well as their regulation after different stimuli, including natural or experimental infections, and/or different biotic or abiotic stressors. Overall, functional omics tools provide multifaceted applications ranging from monitoring host's physiology, microbial communities, to optimizing feed formulations and evaluate more in deep feed additives for aquaculture [38]. In this thesis, the microarray approach was selected in order to provide insights into the immune-related transcriptomic profile of the mucosal tissues of interest.

To gain greater biological insight on the transcriptional regulation that might be modulated by functional diets, various analyses can be performed, such as: a) determining the enrichment of known biological functions, interactions or pathways; b) identifying genes' involvement in pathways or networks by grouping genes together based on similar trends; and c) using global changes in gene expression, such as by visualizing all significantly up- or down-regulated genes in the context of the experimental setting. For this purpose, several bioinformatics tools for functional analysis of gene transcriptomic data have being available in the last years [146].

Furthermore, the combination of omics tools and other complementary analysis, such histochemistry, mucus and serum metabolites description, mucus and serum antibacterial activity evaluation, measurement of enzymatic activity, microbiota characterization, pathogenic and stress challenges, among others, allows to contextualize the physiological and immunological responses that may be obtained through gene expression functional analyses into the *in vivo* scenario. This complementary analysis may provide relevant clues on the mechanisms underlying the immunostimulatory properties of feed additives under development for commercial purposes.

Under this context, in this thesis the following methodological approaches have been used for further characterization and validation of the phytogenics-based additive of interest (*Figure 10*).

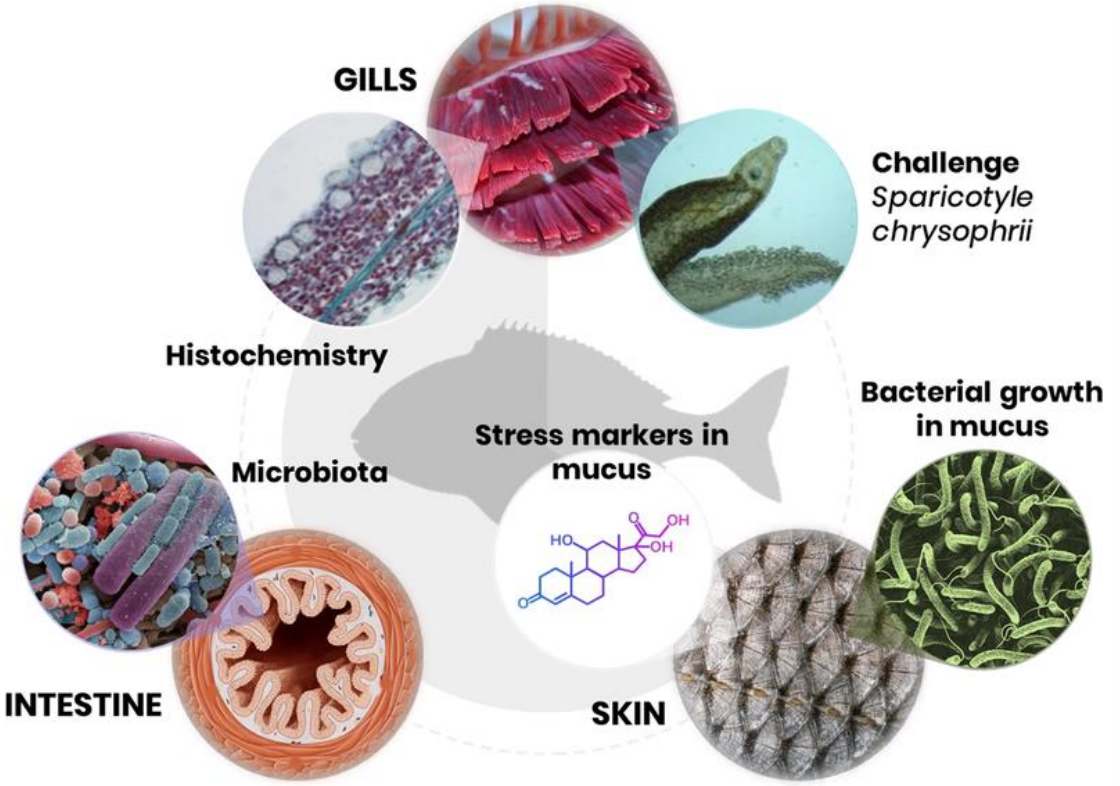


Figure 10: Summary of the complementary analysis performed in the gills, intestine and skin of gilthead seabream (*Sparus aurata*) in order to provide complementary evidence to the transcriptional data in the evaluation of effect of the tested functional feed additive upon mucosal tissues.

In summary, the overall evaluation of the feed additive upon each of the target mucosal tissues was determined by: 1) a microarray-based transcriptomic analysis of the gills, intestine and skin; 2) a functional enrichment analysis to identify classes of over-represented genes that may have an association with particular biological responses; and 3) the application of complementary methodology in order to support the molecular study (Chapter I, II and III). Regarding the complementary methodologies used, these varied depending on the target tissue considered. For instance, a parasitic co-habitation challenge was applied for the gills' study, as well as a histochemical analysis of mucins produced by branchial mucous cells (Chapter I). Secondly, a microbiota analysis based on 16S rRNA sequencing was conducted to evaluate the potential effects of the tested phytogenics on the microbiota

composition and its modulation, and subsequent gut health, as one of the main performance indicators of animal welfare (Chapter II). On the other hand, the antibacterial capacity of the skin mucus was evaluated in order to assess the protective effects of the tested feed additive against other potential pathogens. As indicators of fish welfare, stress-related markers were also measured in the skin mucus as a less-invasive strategy for determining the fish health status (Chapter III).

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OBJECTIVES

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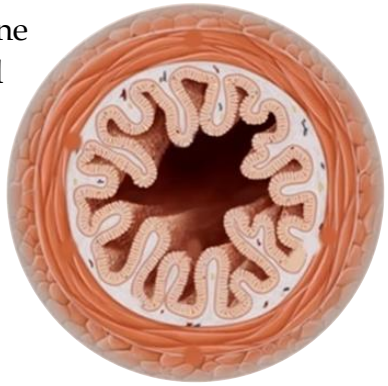
General objective

- To study and characterize the effects of a microencapsulated combination of phytogenic feed additives (garlic essential oil, carvacrol and thymol) upon the mucosal tissues – gills, intestine and skin – in gilthead seabream, as well as its effectiveness against common pathogens, by means of complementary analysis approaches.

Specific objectives

- To determine the effect of the administration of the phytogenics-supplemented diet on gilthead seabream growth parameters during the on-growing stage.
- To describe the gills' transcriptional immune response to the phytogenics-supplemented diet administration;
- To describe the gill's histochemical alterations at cellular level promoted by the tested functional additive;
- To evaluate the antiparasitic properties of the tested functional diet *in vivo* against the major parasitic pathogen affecting farmed gilthead seabream, *Sparicotyle chrysophrii*.

- To describe the intestine transcriptional immune response to the phytogenics-supplemented diet administration;



- To analyse the impact of the phytogenics-supplemented diet administration on the composition and functionality of the intestinal microbiota in gilthead seabream.

- To describe the skin transcriptional immune response to the phytogenics-supplemented diet administration;

- To evaluate the protective effect of the tested functional diet in the gilthead seabream skin mucus against the bacterial growth of two of the major pathogens affecting farmed gilthead seabream, *Vibrio anguillarum* and *Pseudomonas anguilliseptica*;

- To evaluate the effect of the tested functional diet upon the fish allostatic load, through the measurement of stress-related markers present in the skin mucus of gilthead seabream.

- To provide insights into the molecular and cellular mechanisms underlying the immunostimulatory effects promoted by the tested phytogenics-supplemented diet.

CHAPTER I

Unveiling the effect of dietary essential oils supplementation in *Sparus aurata* gills and its efficiency against the infestation by *Sparicotyle chrysophrii*

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Unveiling the effect of dietary essential oils supplementation in *Sparus aurata* gills and its efficiency against the infestation by *Sparicotyle chrysophrii*

Joana P. Firmino[1,2,3], Eva Vallejos-Vidal[4], Carmen Sarasquete[5], Juan B. Ortiz-Delgado[5], Joan Carles Balasch[6], Lluis Tort[6], Alicia Estevez[1], Felipe E. Reyes-López[6]✉ & Enric Gisbert[1]✉

A microencapsulated feed additive composed by garlic, carvacrol and thymol essential oils (EOs) was evaluated regarding its protective effect in gills parasitized by *Sparicotyle chrysophrii* in *Sparus aurata*. A nutritional trial (65 days) followed by a cohabitation challenge with parasitized fish (39 days) were performed. Transcriptomic analysis by microarrays of gills of fish fed the EOs diet showed an up-regulation of genes related to biogenesis, vesicular transport and exocytosis, leukocyte-mediated immunity, oxidation–reduction and overall metabolism processes. The functional network obtained indicates a tissue-specific pro-inflammatory immune response arbitrated by degranulating acidophilic granulocytes, sustained by antioxidant and anti-inflammatory responses. The histochemical study of gills also showed an increase of carboxylate glycoproteins containing sialic acid in mucous and epithelial cells of fish fed the EOs diet, suggesting a mucosal defence mechanism through the modulation of mucin secretions. The outcomes of the in vivo challenge supported the transcriptomic results obtained from the nutritional trial, where a significant reduction of 78% in the abundance of *S. chrysophrii* total parasitation and a decrease in the prevalence of most parasitic developmental stages evaluated were observed in fish fed the EOs diet. These results suggest that the microencapsulation of garlic, carvacrol and thymol EOs could be considered an effective natural dietary strategy with antiparasitic properties against the ectoparasite *S. chrysophrii*.

Nutritional therapies provide an important strategy for preventing and/or treating diseases[1]. Among different options, such as the use of probiotics, prebiotics, immunostimulants and organic acids, phytogenics have gained interest as feed additives within aquafeeds[2]. Phytogenics are plant-based natural substances derived from herbs, spices or extracts similar to essential oils (EOs), which are reputed for their beneficial properties and efficacy on performance and health in animal production[3]. In aquafeeds, EOs as dietary additives have been reported to stimulate appetite, improve feed utilization and growth, and boost the innate immunity[4].

Thymol, carvacrol, cinnamaldehyde and EOs from clove, coriander, star anise, ginger, garlic, rosemary, mint among others, have been used either individually or as blends in animal nutrition[5,6]. Among phytogenics, oregano (*Origanum vulgare*) is the most common because of its richness in carvacrol and thymol[7,8]. These compounds have a wide range of properties such as antimicrobial[9], immunostimulant and anti-oxidative activities[10,11], and the ability to enhance intestinal absorption[12], to improve growth[13] and even to reduce cumulative mortality[11]. The effectiveness of garlic (*Allium sativum*) extract as an immunostimulant, antimicrobial and antiparasitic agent

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	Nutritional trial					
	Day 0		Day 65		Day 104	
Diets	Control	EOs	Control	EOs	Control	EOs
BW (g)	40.2 ± 4.7	40.4 ± 5.1	157.8 ± 14.2	150.8 ± 14.9	205.4 ± 23.9	195.4 ± 21.7
SL (cm)	11.8 ± 0.4	11.9 ± 0.5	17.3 ± 0.6	17.1 ± 0.6	18.3 ± 0.5	18.2 ± 0.4
K	2.3 ± 0.2	2.4 ± 0.1	3.1 ± 0.1	3.0 ± 0.1	3.2 ± 0.2	3.1 ± 0.2
SGR_{BW} (%)	–	–	2.12 ± 0.07	2.03 ± 0.01	1.60 ± 0.18	1.52 ± 0.17
SR (%)			92	96	100	100
			Cohabitation trial			

Table 1. Body weight (BW, standard length (SL), Fulton's condition index (K), specific growth rate for body weight (SGR_{BW}) (mean ± SD) and survival rate (SR) of juvenile gilthead seabream fed with the control and garlic, carvacrol and thymol essential oils (EOs) experimental diets at the beginning of the nutritional trial (day 0), at the beginning of the cohabitation trial with *S. chrysophrii* (day 65) and at the end of the study (day 104). No significant differences were observed between dietary groups ($P > 0.05$).

has been demonstrated in several fish species[14–18]; the inclusion of garlic extract in fish diets is effective against monogenean parasites[19,20].

Combinations of different EOs are promising strategies for functional feeds; however, evaluating their effectiveness in front of a biological challenge such as an ectoparasite infestation, as well as deciphering their mode of action is necessary *prior* to their recommendation as feed additives[21]. Under this context, the aim of this work was to evaluate the functional response of gilthead seabream, the most important farmed fish in the Mediterranean basin, to the dietary administration of a microencapsulated combination of garlic, carvacrol and thymol EOs. In teleosts, gills are one of the main mucosal barriers containing an associated-lymphoid tissue (GIALT) with innate and adaptive immune components that pathogens encounter upon first contact with the host[22,23]. Thus, we ran a nutritional trial with the assessment of gill's transcriptomic profiling in order to describe for the first time the main metabolic and immune pathways regulated by these EOs in this lymphoid tissue, as well as the histochemical properties of mucins produced by branchial mucous cells. Moreover, the efficiency in controlling the infestation by *S. chrysophrii* was also assessed through an in vivo cohabitation challenge trial.

Results

Growth performance. At the end of the nutritional period or at the end of the *S. chrysophrii* challenge, no differences in SR, BW, SL, K or SGR_{BW} were found between fish fed both diets (Table 1; $P > 0.05$).

Gill's transcriptomic profile. A microarray-based transcriptomic analysis was conducted to determine the modulatory effect of dietary EOs upon the gill's transcriptome in healthy fish. In total, 759 DEGs ($P < 0.05$; Supplementary Table 1) were found in gills comparing both groups. From these, 556 genes were up-regulated with 551 mainly concentrated in the 1.0- to 1.5-fold change (FC) interval. The other 5 DEGs were grouped in the $1.5 \leq FC \leq 2.0$ interval. In contrast, 203 genes were down-regulated ($P < 0.05$) and grouped in the range $-1.5 \leq FC \leq -1.0$ (Fig. 1). These results indicated that genes were mostly up-regulated in fish fed dietary EOs and their modulation was moderated in terms of fold-change intensity.

Regarding the total DEGs, 367 nodes generated a functional network in the transcripteractome, resulting in 1171 interactions/edges (average node degree: 6.38; average local clustering coefficient: 0.359; PPI enrichment $P < 1.0\ e^{-16}$). The remaining 392 DGEs were annotated as unknown genes. From the enrichment analysis, five main representative processes (biogenesis, vesicle-mediated transport, immunity, oxidation–reduction, and metabolism; Fig. 2) were identified in the transcripteractome (Supplementary Table 2).

The biological processes associated to biogenesis in the gills were favoured by dietary EOs (34 up-regulated genes; 2 down-regulated genes) (Fig. 3). Several biological processes were identified within the biogenesis process context, namely "translation" (GO:0006412; 19 up-regulated genes; 1 down-regulated gene), "translational elongation" (GO:0006414; 8 up-regulated genes; 1 down-regulated gene), "rRNA processing" (GO:0006364; 11 up-regulated genes; 0 down-regulated genes), "ribosome biogenesis" (GO:0042254; 18 up-regulated genes; 1 down-regulated gene), "ribosomal large subunit export from nucleus" (GO:0000055; 2 up-regulated genes; 1 down-regulated gene) and "peptide biosynthetic process" (GO:0043043; 21 up-regulated genes; 1 down-regulated gene).

Metabolism-related processes were favoured by dietary EOs (132 up-regulated genes; 28 down-regulated genes). In agreement with biogenesis-related processes, the "peptide metabolic process" (GO:0006518; 28 up-regulated genes; 0 down-regulated genes) was also positively regulated in the gills of fish fed the EOs diet, among other processes such as the "regulation of protein metabolic process" (GO:0051246; 55 up-regulated genes; 18 down-regulated genes), "cellular protein metabolic process" (GO:0044267; 77 up-regulated genes; 17 down-regulated genes) and "cellular lipid metabolic process" (GO:0044255; 30 up-regulated genes; 3 down-regulated genes), which were expressed as shown in Fig. 4.

Genes associated with vesicular transport were positively regulated (72 up-regulated genes; 19 down-regulated genes) to dietary EOs (Fig. 5). Some of the GO were identified as representative such as "vesicle-mediated transport" (GO:0016192; 48 up-regulated genes; 15 down-regulated genes), "exocytosis" (GO:0006887; 30 up-regulated

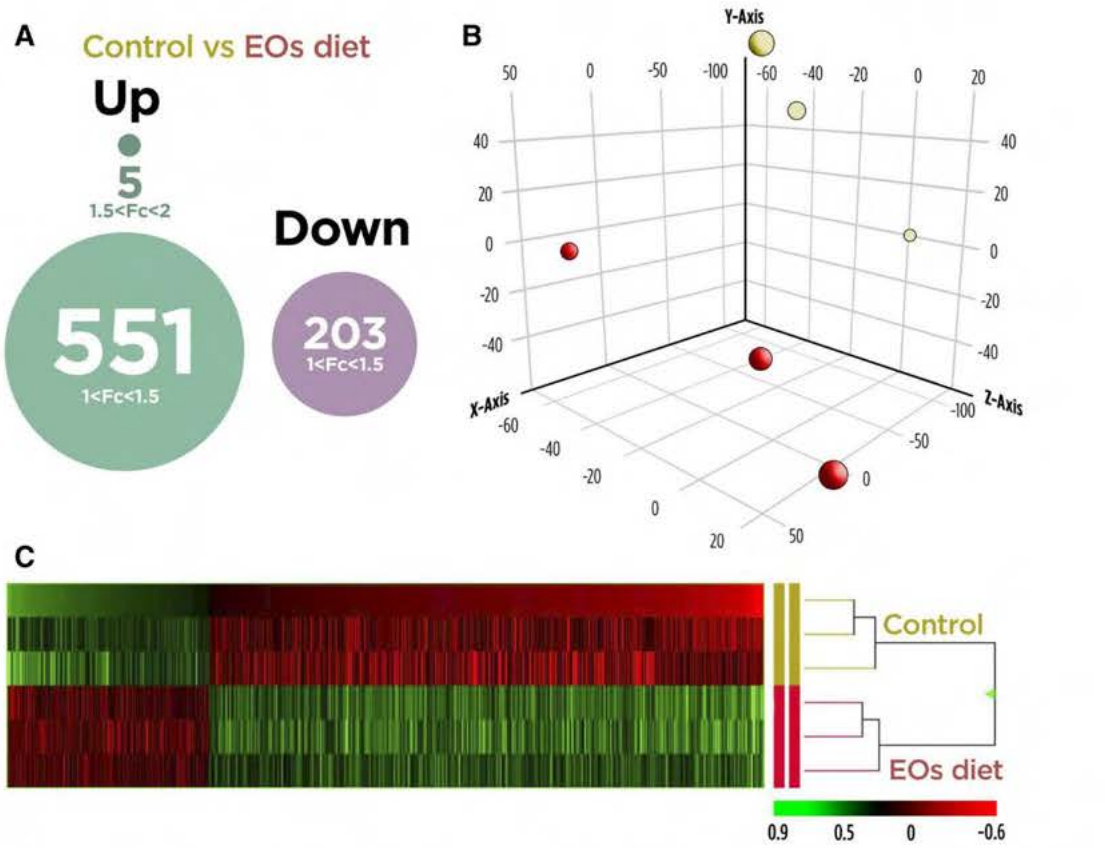


Figure 1. (**A**) Differential expression analysis of the gilthead seabream gill's transcriptomic response to the garlic, carvacrol and thymol essential oils (EOs) supplemented diet. Comparing both groups, 759 DEGs ($P < 0.05$) were found. From these, 556 genes were up-regulated: 551 mainly concentrated in the 1.0- to 1.5-fold change (FC) interval; and 5 DEGs were grouped in the $1.5 \leq FC \leq 2.0$ interval. Additionally, 203 genes were down-regulated ($P < 0.05$) and grouped in the range $-1.5 \leq FC \leq -1.0$. (**B**) Principal component analysis (PCA) of the DEGs of gilthead seabream gill's response to the control diet and EOs supplemented diet. (**C**) Hierarchical clustering of the gilthead seabream gill's transcriptomic response for the control diet and EOs supplemented diet, based in similitude patterns of the differentially expressed genes (DEGs) detected from three sample pools per dietary group. Data of the six microarrays are depicted, one for each represented pool. Both increased and decreased gene expression pattern is shown in green and red, respectively. All transcripts represented are statistically significant ($P < 0.05$).

genes; 10 down-regulated genes), "protein transport" (GO:0015031; 44 up-regulated genes; 11 down-regulated genes), "intracellular protein transport" (GO:0046907; 31 up-regulated genes; 7 down-regulated genes) and "endoplasmic reticulum to Golgi vesicle-mediated transport" (GO:0006888; 9 up-regulated genes; 3 down-regulated genes).

Biological processes related to an immune effector response showed an important up-regulation response in gills (29 up-regulated genes; 8 down-regulated genes) of fish fed dietary EOs (Fig. 6). Some GO related with immunity were highlighted such as "leukocyte-mediated immunity" (GO:0002443; 24 up-regulated genes; 5 down-regulated genes), "leukocyte activation" (GO:0045321; 29 up-regulated genes; 8 down-regulated genes), "myeloid leukocyte activation" (GO:0002274; 23 up-regulated genes; 5 down-regulated genes), "neutrophil-mediated immunity" (GO:0002446; 23 up-regulated genes; 5 down-regulated genes) and "neutrophil degranulation" (GO:0043312; 22 up-regulated genes; 5 down-regulated genes). Particularly, the neutrophil-mediated immunity process shared 27 of 28 total regulated genes with the exocytosis process. A set of up-regulated genes related to anti-inflammatory response (*il7*, *il6r*, *il20ra* and *il21r*) were detected as mediators of immunity processes. Another relevant biological process positively affected by the dietary inclusion of EOs was the "oxidation–reduction process" (GO:0055114; 43 up-regulated genes and 4 down-regulated genes9 (Fig. 7).

Histological organization and gill's histochemistry. No major differences in the histological organization of gills in fish from both experimental groups were observed (consult Feist[24] for details on gill's histological organization). Gills of fish fed the control diet showed no histochemical differences between the mucous cells (MCs) of primary and secondary gill lamellae. Nonetheless, the number of MCs was lower in the secondary lamellae (*ca.* 40 MCs mm^{-1}) and more abundant in the epithelial and opercular areas, where they were intensely stained with most of histochemical and lectin techniques performed (Table 2). In brief, most of MCs from the

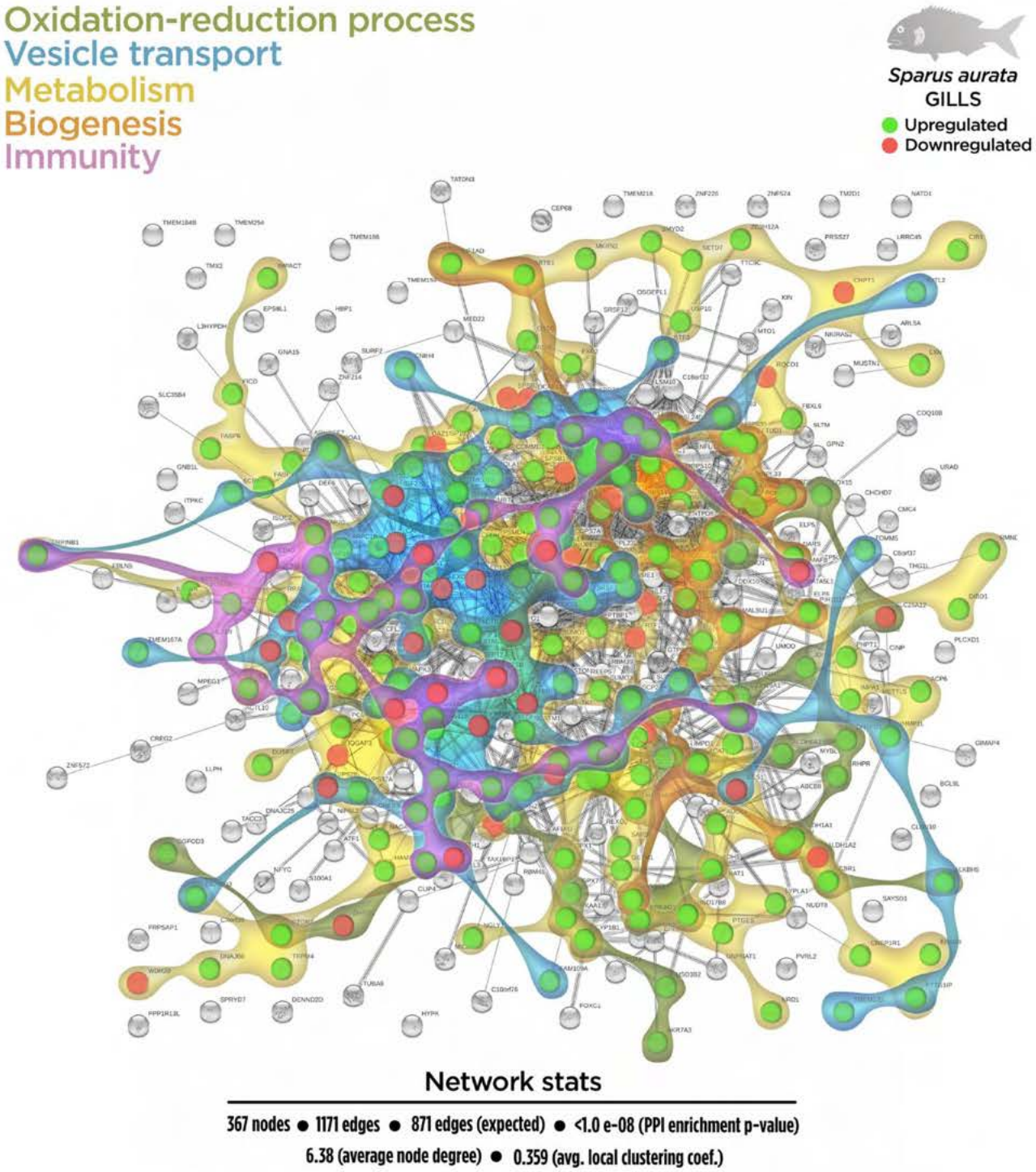
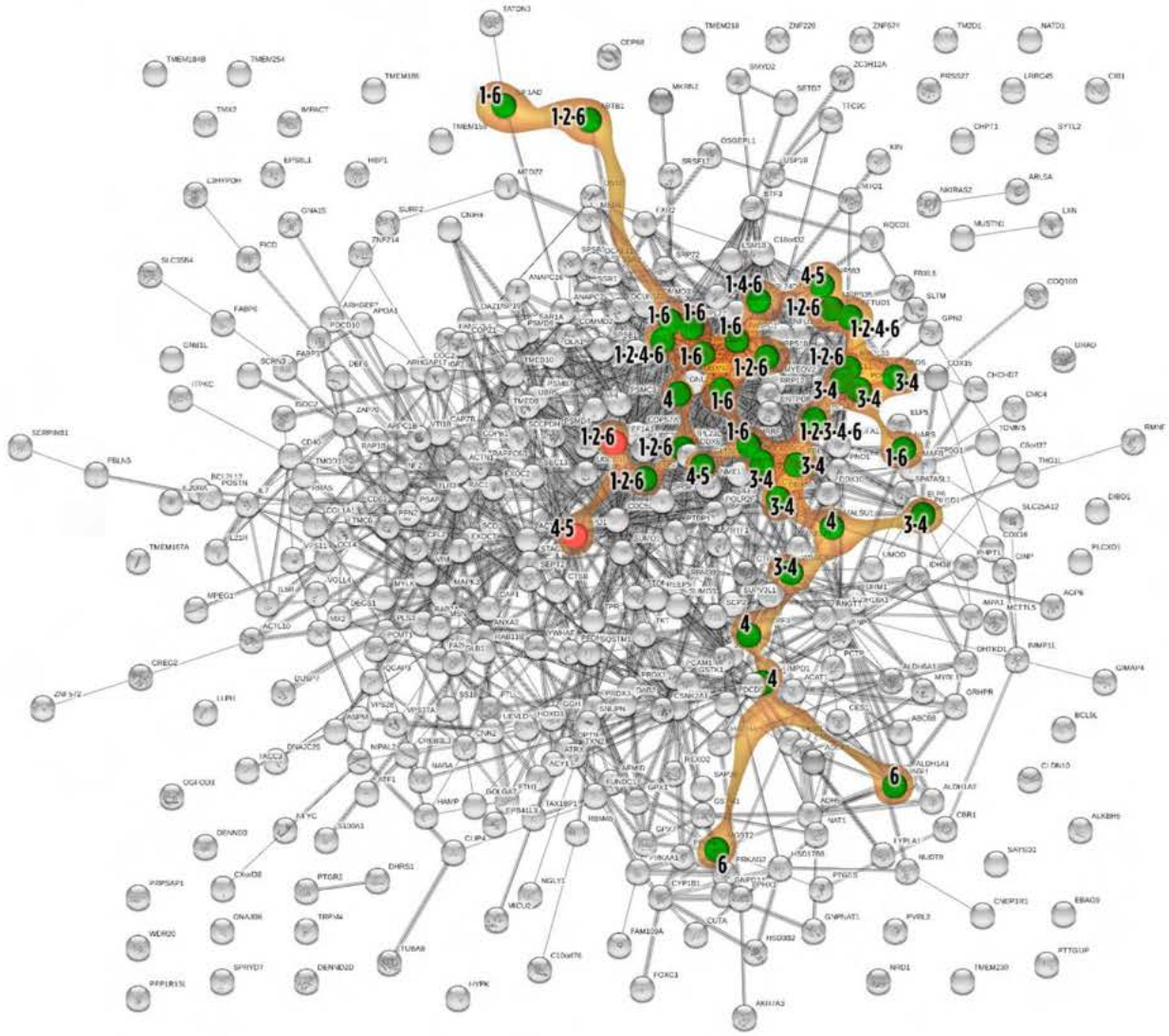


Figure 2. Transcripteractome of the differentially expressed genes (DEGs) in the gills of juvenile gilthead seabream fed the garlic, carvacrol and thymol essential oils (EOs) supplemented diet. Five representative processes identified from the functional enrichment analysis—biogenesis, metabolism, vesicle-mediated transport, immunity and oxidation–reduction—are highlighted distinctly in coloured amoeboid clusters in the overall Protein–Protein Interactions Network (PPI) for the DEGs (see also Supplementary Table 2). Green nodes represent up-regulated genes and red nodes represent down-regulated genes. Graphic keys including colours and network stats are indicated in the graphical figure legend.

control group contained abundant glycoproteins (GPs) with oxidizable vicinal diols groups (KOH-PAS), indicating the presence of neutral GPs, as well as in the epithelial cell layer due to the presence of secreted mucins. A scarce number of MCs contained GPs with O-sulphate esters (AB pH 1.0), whereas GPs with or without *O*-acyl

Biogenesis

Go-term	Description	Count in gene set	False discovery rate
GO:0006412	**1** translation	19 of 362	0.0059
GO:0006414	**2** translational elongation	9 of 116	0.0235
GO:0006364	**3** rRNA processing	11 of 192	0.0462
GO:0042254	**4** ribosome biogenesis	19 of 270	0.00056
GO:0000055	**5** ribosomal large subunit export from nucleus	3 of 8	0.0354
GO:0043043	**6** peptide biosynthetic process	21 of 386	0.0023



Network stats

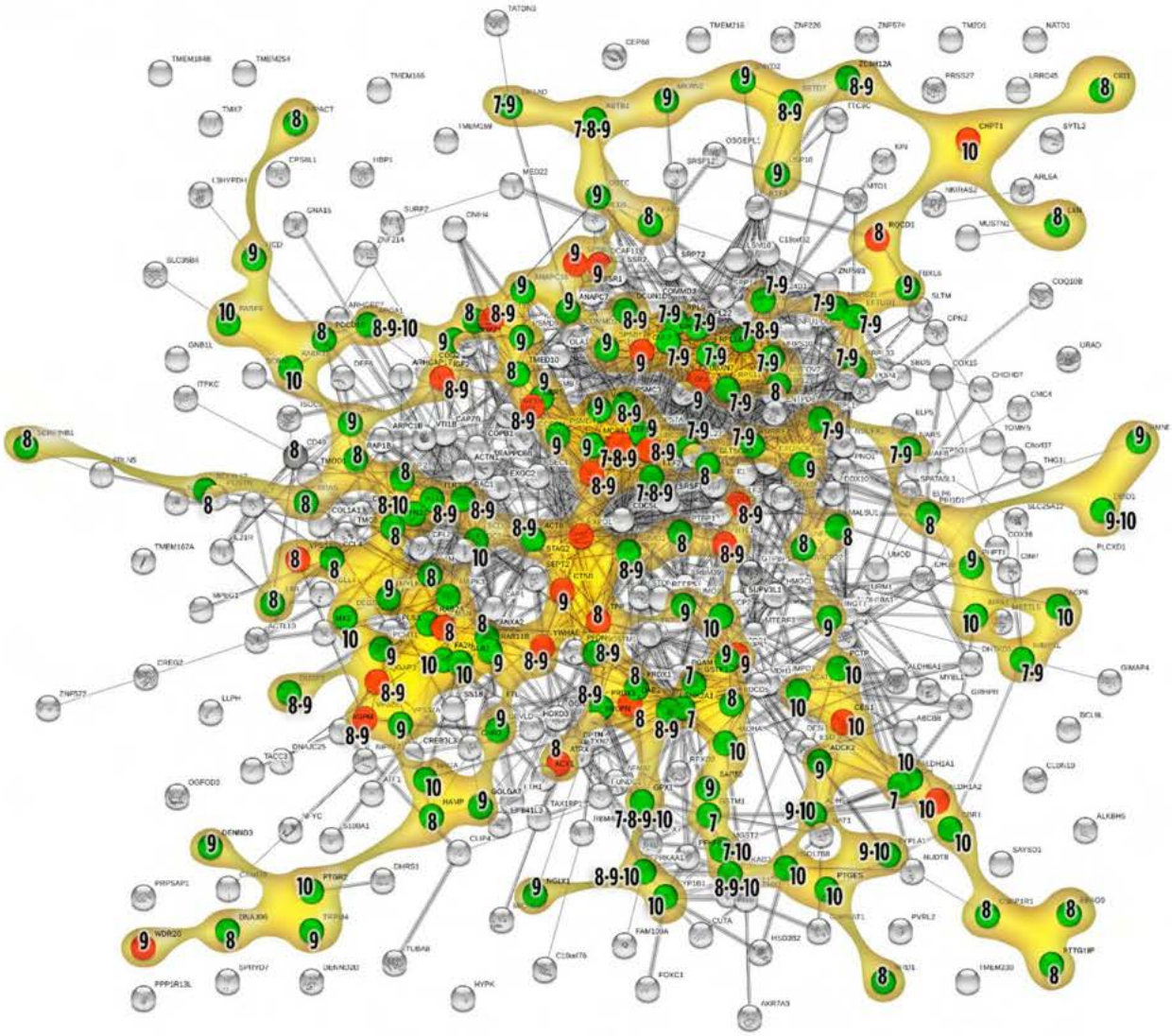
367 nodes ● 1171 edges ● 871 edges (expected) ● <1.0 e-08 (PPI enrichment p-value)

6.38 (average node degree) ● 0.359 (avg. local clustering coef.)

Figure 3. Biogenesis-related Protein–Protein Interactions Network (PPI) network for the differentially expressed genes (DEGs) in the gills of juvenile gilthead seabream fed the garlic, carvacrol and thymol essential oils (EOs) supplemented diet (see also Supplementary Table 2). Nodes numbers (1–6) indicate the biological processes for each DEG represented. Gene Ontology (GO) definitions, count of DEGs within each biological processes and respective false discovery rate are described in the graphical figure legend. Green nodes represent up-regulated genes and red nodes represent down-regulated genes. Graphic keys and network stats are indicated in the graphical figure legend.

Metabolism

Go-term	Description	Count in gene set	False discovery rate
GO:0006518	**7** peptide metabolic process	26 of 497	0.0010
GO:0051246	**8** regulation of protein metabolic process	72 of 2668	0.0382
GO:0044267	**9** cellular protein metabolic process	92 of 3603	0.0383
GO:0044255	**10** cellular lipid metabolic process	32 of 946	0.0421



Network stats

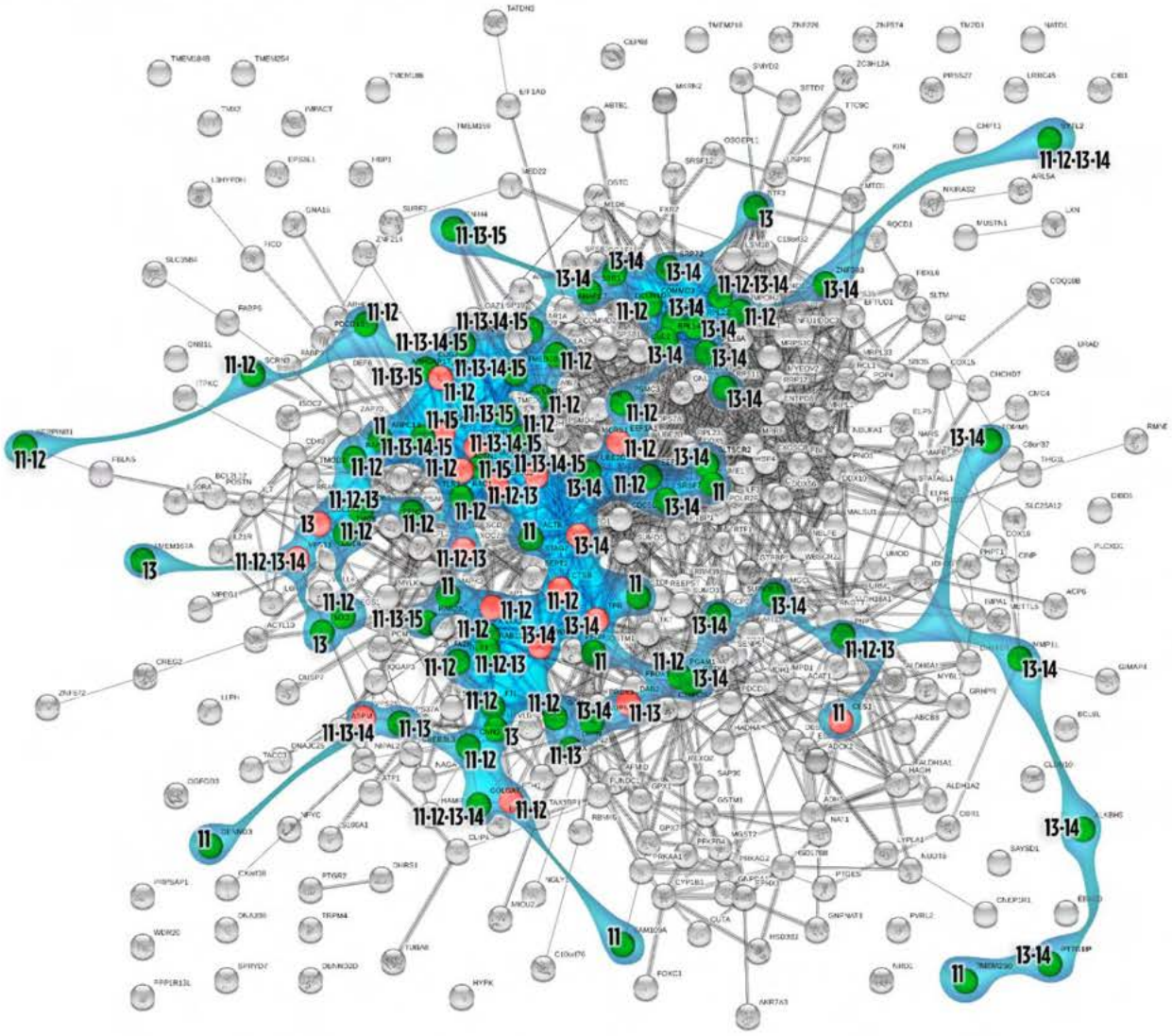
367 nodes ● 1171 edges ● 871 edges (expected) ● <1.0 e-08 (PPI enrichment p-value)

6.38 (average node degree) ● 0.359 (avg. local clustering coef.)

Figure 4. Metabolism-related Protein–Protein Interactions Network (PPI) network for the differentially expressed genes (DEGs) in the gills of juvenile gilthead seabream fed the garlic, carvacrol and thymol essential oils (EOs) supplemented diet (see also Supplementary Table 2). Nodes numbers (7–10) indicate the biological processes for each DEG represented. Gene Ontology (GO) definitions, count of DEGs within each biological processes and respective false discovery rate are described in the graphical figure legend. Green nodes represent up-regulated genes and red nodes represent down-regulated genes. Graphic keys and network stats are indicated in the graphical figure legend.

Vesicle transport

Go-term	Description	Count in gene set	False discovery rate
GO:0016192	**11** vesicle-mediated transport	62 of 1699	0.00026
GO:0006887	**12** exocytosis	39 of 774	3.70e-05
GO:0015031	**13** protein transport	51 of 1391	0.0010
GO:0006886	**14** intracellular protein transport	34 of 836	0.0027
GO:0006888	**15** ER to Golgi vesicle-mediated transport	12 of175	0.0107



Network stats

367 nodes ● 1171 edges ● 871 edges (expected) ● <1.0 e-08 (PPI enrichment p-value)

6.38 (average node degree) ● 0.359 (avg. local clustering coef.)

Figure 5. Vesicle-mediated transport Protein–Protein Interactions Network (PPI) network for the differentially expressed genes (DEGs) in the gills of juvenile gilthead seabream fed the garlic, carvacrol and thymol essential oils (EOs) supplemented diet (see also Supplementary Table 2). Nodes numbers (11–15) indicate the biological processes for each DEG represented. Gene Ontology (GO) definitions, count of DEGs within each biological processes and respective false discovery rate are described in the graphical figure legend. Green nodes represent up-regulated genes and red nodes represent down-regulated genes. Graphic keys and network stats are indicated in the graphical figure legend.

Immunity

Go-term	Description	Count in gene set	False discovery rate
GO:0002443	**16** leukocyte mediated immunity	28 of 632	0.0033
GO:0045321	**17** leukocyte activation	35 of 894	0.0040
GO:0002274	**18** myeloid leukocyte activation	27 of 574	0.0022
GO:0002446	**19** neutrophil mediated immunity	27 of 498	0.00055
GO:0043312	**20** neutrophil degranulation	26 of 485	0.00085

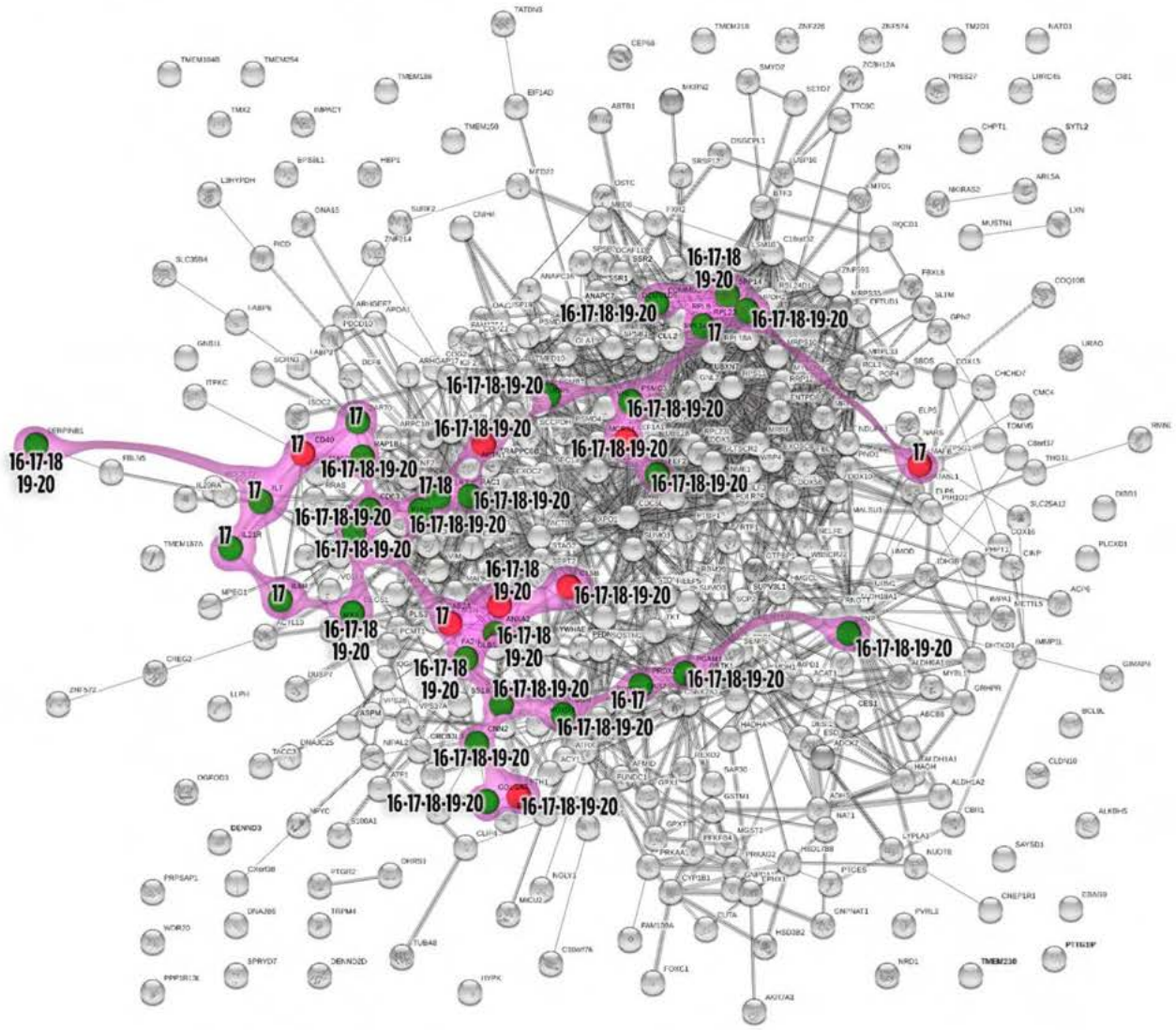


Figure 6. Immunity-related Protein–Protein Interactions Network (PPI) network for the differentially expressed genes (DEGs) in the gills of juvenile gilthead seabream fed the garlic, carvacrol and thymol essential oils (EOs) supplemented diet (see also Supplementary Table 2). Nodes numbers (16–20) indicate the biological processes for each DEG represented. Gene Ontology (GO) definitions, count of DEGs within each biological processes and respective false discovery rate are described in the graphical figure legend. Green nodes represent up-regulated genes and red nodes represent down-regulated genes. Graphic keys and network stats are indicated in the graphical figure legend.

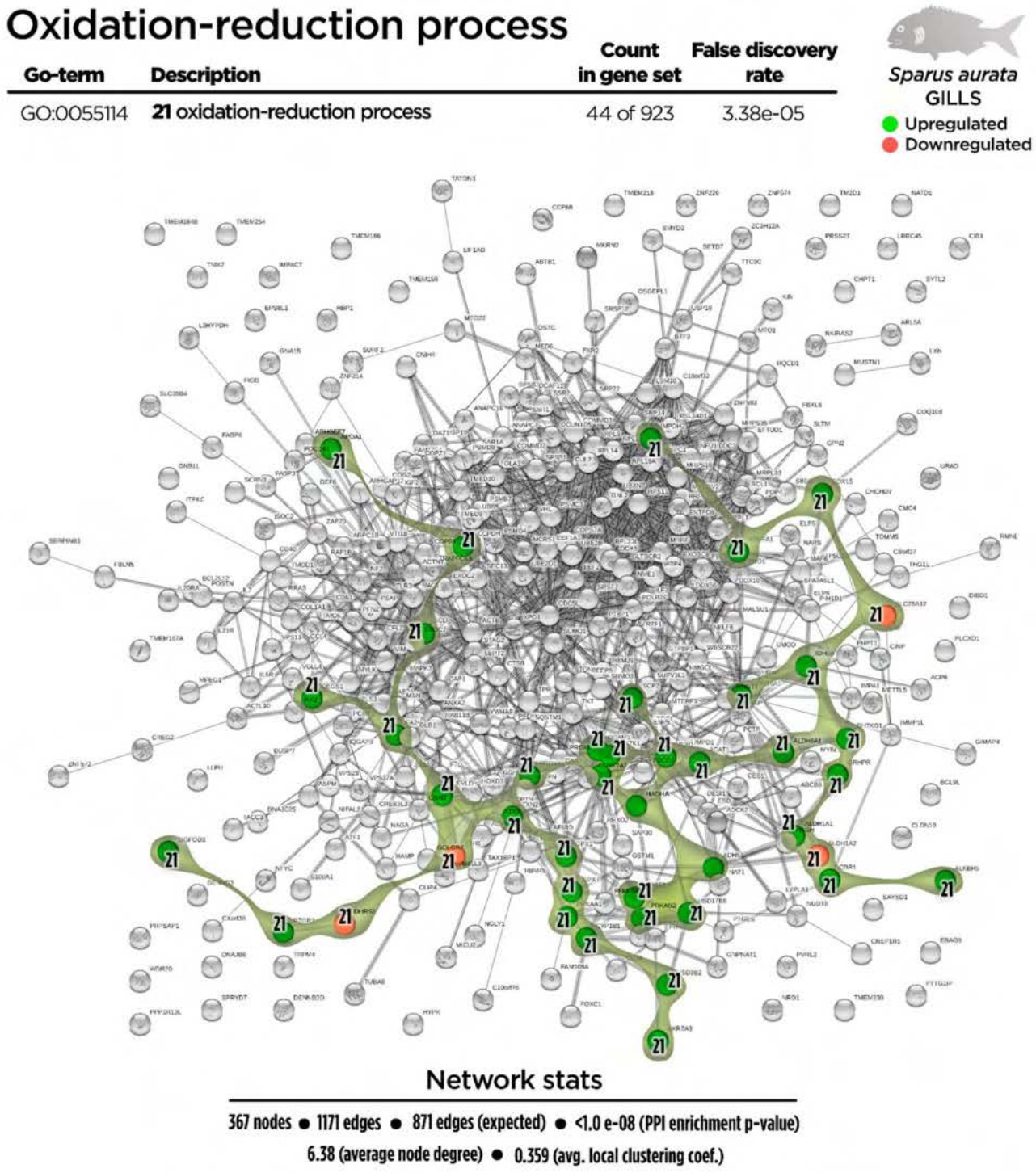


Figure 7. Oxidation–reduction Protein–Protein Interactions Network (PPI) network for the differentially expressed genes (DEGs) in the gills of juvenile gilthead seabream fed the garlic, carvacrol and thymol essential oils (EOs) supplemented diet (see also Supplementary Table 2). Nodes number (21) illustrate the oxidation–reduction biological process for each DEG represented. Gene Ontology (GO) definition, count of DEGs and respective false discovery rate are described in the graphical figure legend. Green nodes represent up-regulated genes and red nodes represent down-regulated genes. Network stats are indicated in the graphical figure legend.

sugars (KOH-PAS) were also registered in moderate or high amounts. Most MCs were strongly stained with PAS and diastase-PAS (absence of glycogen), whereas some of these cells containing neutral GPs also displayed a strong alcianophilia (AB pH 2.5), evidencing the presence of carboxylated GPs. Regarding lectins, MCs and the epithelial cell layers from the control group displayed from weak to moderate or strong affinity to several

Diets	Epithelial cell layer (secreted mucins)		Mucous cells (non-secreted mucins)	
	Control	EOs	Control	EOs
General histochemistry				
Schiff	0	0	0	0
PAS	2	2	2	2
Diastase-PAS	2	2	2	2
KOH-PAS	2	2	3	3
Alcian-Blue (AB) pH 2.5	1	2	2	3
Alcian Blue pH 1	1	2	1	1
Alcian Blue pH 0.5	0–1	1	0	0
Neuraminidase-AB pH 2.5	0–1	1	1	1
Lectin histochemistry				
ConA	1	1	1	1
UEA-I	1	1	0	0
WGA	2	3	2	3
SNA	0–1	1	0–1	1
SBA	2	2	2	2

Table 2. Histochemical properties of the mucous cells and the epithelium in gills from gilthead seabream fed a the control diet and the diet supplemented with a blend of garlic, carvacrol and thymol essential oils (EOs). Results are expressed as the semiquantitative assessment of colour intensities by the scores of four independent observers: (0) negative; (1) weak; (2) moderate; (3) intense; and (4) very intense. *PAS* Periodic Acid Schiff, *AB* Alcian Blue, *ConA* Concanavalin A, *UEA-I Ulex europeus* agglutinin, *WGA* Wheat germ agglutinin, *SNA Sambucus nigra* lectin, *SBA* Soybean agglutinin.

tested lectins (ConA, WGA, UEA-I, SNA and SBA) indicating the differential presence of Man/Glc, βGlcNAc, NeuNAc/sialic acids/NANA and GalNAc residues in mucins. The presence of GPs containing fucose residues was rare, being only evidenced in epithelial cell layers. In addition, the decreased reactivity after neuraminidase treatment before AB pH 2.5 and the presence of WGA and SNA lectins, indicated the occurrence of both non-acetylated and acetylated sialic acids in mucins.

Dietary EOs promoted modifications on the histochemical properties of MCs and their secretions. Although the number of MCs did not change, most of these secretory cells were hypertrophied ($158.7 \pm 9.9\ \mu m^2$) when compared with the control group ($91.7 \pm 9.2\ \mu m^2$) (Fig. 8). The most noticeable histochemical effect was the increase of carboxylated GPs (AB pH 2.5) containing sialic acid. In addition, residues of βGlcNAc and NeuNAc/sialic acids/NANA (WGA, SNA lectins) in the glycoconjugate contents of MCs and branchial epithelia were observed at higher intensity levels (Fig. 8, Table 2).

Ectoparasite challenge. Fish fed dietary EOs had a significantly lower number of total parasite intensity (6.6 ± 4.5 parasite fish^{-1}) and abundance (6.6 ± 4.3 parasite fish^{-1}) when compared to the control group (29.5 ± 15.1 and 27.4 ± 11.3 parasite fish^{-1}, respectively) ($P < 0.05$; Fig. 9), resulting in a reduction of 77.6% of total parasite load. The number of eggs present in fish fed the EOs-diet in terms of intensity (3.6 ± 3.2 parasite fish^{-1}) and abundance (1.2 ± 2.5 parasite fish^{-1}) was lower than in the control group (10.8 ± 12.3 and 9.3 ± 12.0 parasite fish^{-1}, respectively), and prevalence decreased from 80.0% in control group to 33.3% in the EOs group. The number of post-larvae present in fish fed the EOs-diet in terms of intensity (1.6 ± 0.5 parasite fish^{-1}) and abundance (0.7 ± 0.9 parasite fish^{-1}) was not significantly different than in the control group (2.4 ± 1.4 and 2.4 ± 1.4 parasite fish^{-1}, respectively), and prevalence decreased from 93.3% in control group to 46.7% in the EOs group. However, the number of juvenile ectoparasites intensity and abundance was lower in fish fed the additive (4.1 ± 2.2 and 4.1 ± 2.1 parasite fish^{-1}, respectively) in comparison to those fed the control diet (10.9 ± 4.0 and 10.9 ± 3.8 parasite fish^{-1}, respectively), and prevalence was 100% for both experimental groups. The number of adults intensity and abundance was also lower in fish fed the additive (2.7 ± 2.4 and 1.8 ± 2.3 parasite fish^{-1}, respectively) than in those fed the control diet (14.1 ± 7.2 and 14.1 ± 7.0 parasite fish^{-1}, respectively), and prevalence decreased from 100% in control group to 66.7% in the EOs group.

Discussion

The use of garlic, carvacrol and/or thymol EOs in functional aquafeeds has been tested and demonstrated both in vitro and in vivo for its effectiveness in fighting against bacterial and parasitic infections[2,25,26]. The anthelmintic properties of EOs and gill's response to their dietary administration are poorly understood and available information is scarce. Hence, most of the existing studies are focused in the effects of the above-mentioned EOs on intestinal health[10,12,27,28], as well as in their use in balneation treatments against parasitic organisms[15,16,29–31]. As far as we know, this is the first study describing the gill's response in gilthead seabream to the administration of the above-mentioned EOs as a feed additive, as well as the mechanisms underlying its antiparasitic properties.

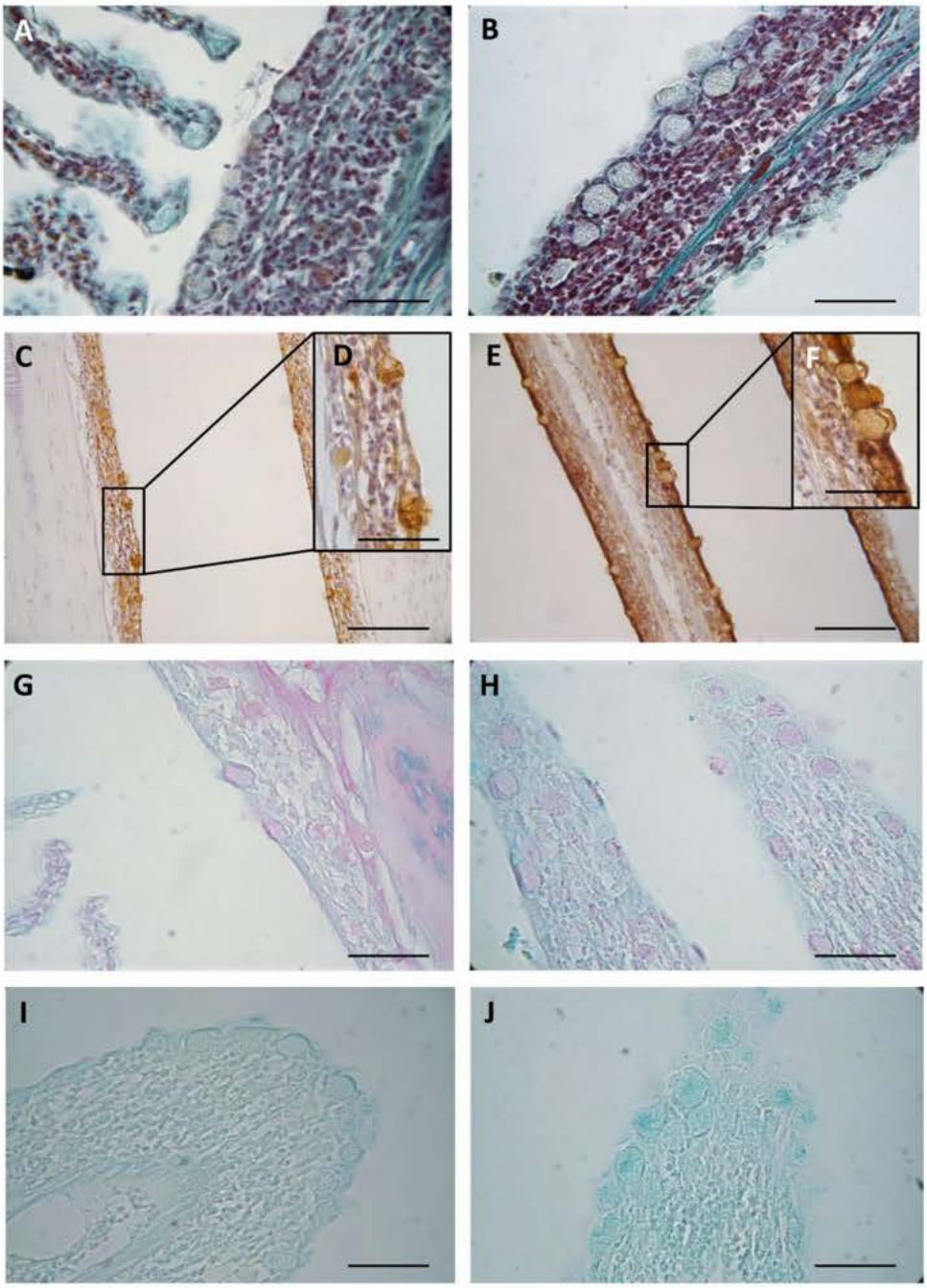
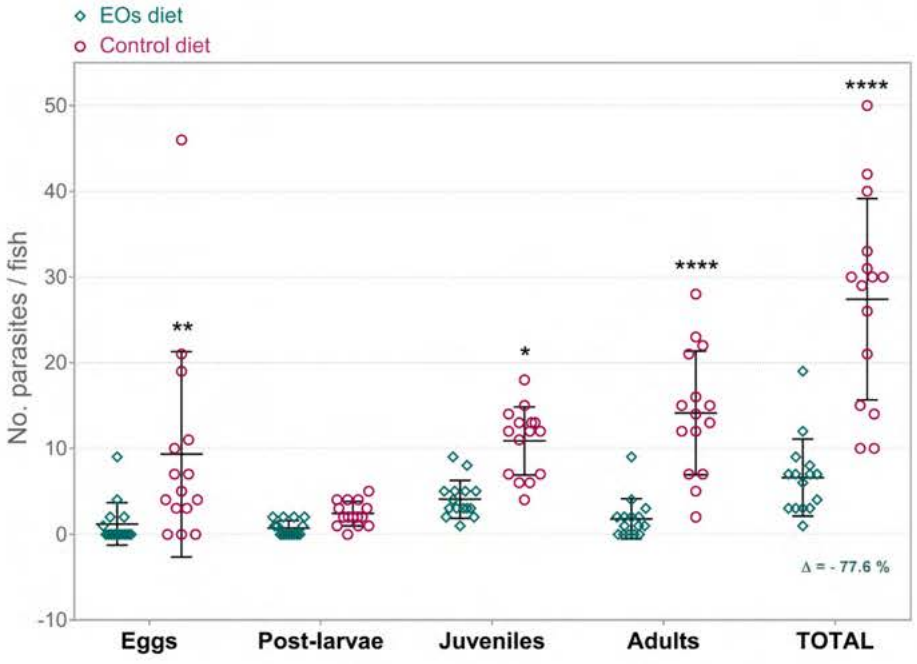


Figure 8. Histological sections from gills of gilthead seabream (*Sparus aurata*) fed the control diet (**A**, **C**, **D**, **G**, **I**) and the diet supplemented with a blend of garlic, carvacrol and thymol essential oils (EOs) (**B**, **E**, **F**, **H**, **I**). Histomorphological detail of mucous cells from control (**A**) and EOs (**B**) diets. Note the hypertrophy of mucous cells in fish fed the EOs diet (**B**) when compared to the size of mucous cells of the control group (**A**) (staining: H/VOF). Mucous cells content rich in *N*-acetyl-D-glucosamine and/or *N*-acetylneuraminic acid/sialic acid in fish fed control (**C**, **D**) and EOs (**E**, **F**) diets. Note increase in staining intensity in both epithelium and mucous cells from EOs diet (staining: WGA lectin). Presence of neutral glycoproteins in branchial mucous cells from control (**G**) and EOs (**H**) diets (staining: PAS). Carboxylated glycoproteins were also detected both in epithelium and mucous cells from control (**I**) and EOs (**J**) diets. Note increased in staining intensity in fish fed the EOs diet comparing to controls (staining: AB pH 2.5). Scale bars represent 25 (**A**, **B**, **D**, **F**, **G**, **H**, **I**, **J**) and 50 (**C**, **E**) µm.

The dietary inclusion of microencapsulated garlic, carvacrol and thymol EOs did not affect fish growth. Contrarily, previous studies suggested them as growth promoters in several fish species[13,25,27,32–38]. Although the reasons explaining such differences are out of scope in this study, the utilization of different EO doses,



S. chrysophrii (mean ± SD)

Diet	N fish	Eggs	Post-larvae	Juveniles	Adults	TOTAL
EOs	15	1.2 ± 2.4	0.7 ± 0.9	4.1 ± 2.1	1.8 ± 2.3	**6.6 ± 4.3**
Prevalence %		33.3	46.67	100	66.7	
Control	15	9.3 ± 11.6	2.4 ± 1.4	10.9 ± 3.8	14.1 ± 7.0	**27.4 ± 11.3**
Prevalence %		80.0	93.3	100	100	
		Two-way ANOVA (Sidak's multiple comparisons test)				
		**	*ns*	*	****	****
Adjusted *P*-value		0.0024	0.9561	0.0164	< 0.0001	< 0.0001

Figure 9. Abundance of *S. chrysophrii* parasites in fish fed with the control diet and the diet supplemented with a blend of garlic, carvacrol and thymol essential oils (EOs). Different ectoparasite developmental stages are represented according to their morphological characteristics: eggs, post-larvae (early juveniles with 2–4 pairs of clamps), juveniles and adults. The total load of the ectoparasite (TOTAL) and the percentage of total abundance decrease among experimental diets is also indicated (Δ). Circles and rhombus represent parasite counting per individual fish (n = 15); mean ± standard deviation are represented. Circles (pink): gilthead seabream fed with control diet; Rhombus (green): gilthead seabream fed with EOs supplemented diet. *, ** and **** indicate significant differences between dietary groups with corresponding adjusted P-values described.

administration length and diet formulation could be among the potential factors that may explain such results. Regardless of the fact that growth has been traditionally considered as one of the main end-points and/or key performance indicators for additive testing, some existing literature might not be trustworthy due to constrains in the reproducibility of the compounds studied[39]. Under this context, the use of natural plant-derivates or extracts may lead to variability and discrepancy of results, as opposed to the utilization of similar synthetic compounds, which favours the reproducibility and robustness of the studies. Moreover, SGR values of gilthead sea bream fed both control (2.1 ± 0.07% BW/day) and the EOs supplemented diet (2.03 ± 0.01% BW/day) compared favourably to those reported by Mongile et al.[40] for this species also reared under summer conditions (1.5 ± 0.1% BW/day).

Gills are one of the main mucosal immune barriers in fish[22,41,42], but they also represent an ideal site for the attachment of ectoparasites[43], potentially inducing a host hypometabolic response, as suggested for *S. chrysophrii* infections[44]. In fact, gills are considered one of the most active tissues in the protein synthesis with a significantly high plasticity in protein metabolism[45]. Our transcriptional study revealed several biological processes associated to biogenesis and metabolic processes, including peptide biosynthesis, and protein and lipid metabolism that were predominantly up-regulated in the gills of gilthead seabream fed dietary EOs. Therefore, EOs would contribute to restore the gill's metabolic rate by increasing protein synthesis. Although gill's protein turnover contribution is not significant to the whole body[45], our transcriptional profile is in accordance with studies that have reported the significant influence of garlic[33,34], carvacrol[46] and thymol[13,35] dietary administration on body and blood protein content upon the protein synthesis and metabolism. Collectively, our results provide new evidence for the biological activity of garlic, carvacrol and thymol, indicating these compounds also promote

the protein synthesis and metabolism at mucosal level, particularly on gills (see text below); thus, increasing the overall activity of this tissue.

The vesicular trafficking processes are intrinsic to the secretory protein biogenesis[47]. Despite the previous evidence reporting an augment in the proteins synthesis[13,32–35,46], the transcriptional regulation of vesicle-mediated transport by the dietary administration of garlic, carvacrol and thymol have not been described in fish to date. Genes involved in protein vesicle-mediated transport, such as the ER to Golgi vesicle-mediated transport process, were also positively regulated in the gills of fish fed dietary EOs, as for example the Secretion Associated Ras-related GTPase 1A (*sar1a*), the Rho Guanine Nucleotide Exchange Factor 7 (*arhgef7*), the Vesicle Transport Through Interaction With T-SNAREs 1B (*vti1b*) and some of the Rab Family of GTPases (*rab11b, rab2a*). The above-mentioned genes are known for their role in cellular trafficking pathways, like the RAB11-coding protein, which is recognized for its localization in recycling endosomes and its role in exocytic trafficking[48]. In vitro studies in mammal cells have associated garlic[49], carvacrol and thymol to vesicle fusion and exocytic processes[50]. Therefore, we may infer that the machinery implied in the activation of biogenic processes observed by dietary EOs is inherent to the activation of processes of secretory protein translocation by vesicles.

As previously mentioned, vesicle trafficking and exocytosis are intimately related processes. In this way, genes like Rab GTPases participate in the regulation of the exocytosis membrane trafficking pathway[51]. In our gill's transcriptional analysis, exocytosis was one of the most positively regulated processes by dietary EOs. This finding is especially relevant since exocytosis is recognized by its important role in the immune response participating in neutrophil function[52], in the immunological synapses between cells[53] and in the cell-mediated cytotoxicity[54]. Remarkably, the tested EOs also positively regulated immune-related biological processes by means of myeloid leukocyte immunity activation. Besides, this response appeared to be orchestrated by neutrophil population, since neutrophil mediated immunity and neutrophil degranulation processes were boosted by dietary EOs. In gilthead seabream, acidophilic granulocytes are functionally equivalent to higher vertebrates neutrophils[55,56] being described as one of main phagocytes of this species[57,58] occurring also in mucosal tissues[59–61]. Neutrophils' granules are reputed for their antimicrobial, proteolytic and potential cytotoxic capacities[52,62], which are synthetized during myeloid cell differentiation, comprising specific protein biosynthesis and the early formation of secretory vesicles[52]. Remarkably, we detected the presence of DEGs associated with protein biosynthesis, vesicular transport and exocytosis (as formerly discussed), which could be in their turn up-regulated due to the immunostimulatory effect of dietary EOs upon acidophilic granulocytes degranulation process. For instance, Mitogen-Activated Protein Kinase 3 gene (*mapk3*) resulted up-regulated in the gills of the EOs diet group, with representation both in protein and lipid metabolic and in the vesicle-mediated transport processes. Mitogen-activated protein kinases (MAPK) are known to be involved on signalling pathways of neutrophil functional response[63,64]. There is evidence of the activation of MAPK by ajoene (an organosulfur compound found in garlic) in the process of apoptosis of human cancer cells[65]. Despite of the evidences of an acidophilic granulocyte-mediated immune response stimulation induced by the bioactive compounds of the EOs tested, the exact bioactive compounds and accurate mechanisms involved in the alleged immunomodulatory and antiparasite effects of these EOs still needs to be deciphered when tested in separate.

In aquaculture relevant species, some studies reported an increase of blood neutrophil number after therapeutic balneation with EOs, which was also effective against monogeneans[29,66,67]. Neutrophils function involves the interplay of many different receptors, ion channels and signalling pathways, such as changes in intracellular Ca^{2+} levels, for instance[68]. Accordingly, garlic EO organosulfur compounds were recently demonstrated to activate human neutrophil functional activity through the activation of Ca^{2+} flux[64], whereas ajoene and allicin were described as potent inhibitors of neutrophil ROS production[64]. Similar results were also attributed to thymol redox properties[69], whereas in fish, dietary carvacrol (0.05%) significantly reduced leukocyte ROS release in seabass[11]. Although neutrophils and other circulating leukocytes have a critical role in the innate immune defence against pathogens, such helminth parasites[70] including monogeneans[71]; and that acidophilic granulocytes have been particularly identified in gilthead seabream gill's response to ectoparasite infections[59]; its activity comprises a significant tissue damage associated to the ROS released during the inflammatory process. For instance, it was proposed that gilthead seabream may control *S. chrysophrii* infection through ROS action produced by immune cells. Although parasite evasion mechanisms were also suggested, this response may potentiate secondary infections if it is not properly controlled[72]. Although it might seem contradictory, the sustenance of self-protective antioxidant mechanisms is vital for the correct functioning of the immune system, preventing oxidative damage by ROS that escort leukocyte activity; particularly in neutrophil-mediated immune response[73]. Outstandingly, these antioxidant properties were also highlighted in the transcriptomic profiling of the gills in fish fed dietary EOs, where several genes involved in oxidation–reduction processes were positively regulated. Aquafeeds containing natural garlic (4%), garlic powder (3.2%) and garlic oil (0.25%) promoted glutathione peroxidase, superoxide dismutase and catalase activities in tilapia serum and liver[33]. In seabass, after cadmium-induced toxicity, the up-regulation of genes coding for these enzymes in the liver improved the antioxidant capacity of individuals fed a diet containing garlic powder (2%)[74]. Similarly, an increased antioxidant activity in rainbow trout fillet was associated to dietary carvacrol (1.2%) or thymol (0.6%)[10], and in channel catfish, a commercial product containing *O. heracleoticum* EO (0.05%) enhanced plasma antioxidant activity[32]. In accordance with the above-mentioned studies, our transcriptomic data showed that dietary EOs promoted the up-regulation of Glutathione Peroxidases (*gpx1, gpx7*) and Glutathione S-transferases (*gstm1, gstk1, mgst2*). Additionally, Mitochondrial Thioredoxin (*txn2*), a key antioxidant protein that participates in the removal of ROS and cytotoxicity[75,76], was also up-regulated by dietary EOs. In our study Peroxiredoxins 1 and 3 (*prdx1, prdx3*) were also up-regulated. Additionally, the up-regulation of the Epoxide Hydrolase 1 (*ephx1*) was promoted by dietary EOs; this enzyme has a detoxifying function, playing an important role in cellular and organ defence against exogenous toxicity compounds[77]. Thus, it appears that tested EOs may exert an important antioxidant

action to counteract the impact of the high amounts of ROS released by the previously referred stimulation of acidophilic granulocyte's activity, evidencing the importance of their joint supplementation.

After a proinflammatory phase starred by neutrophils and their ROS production, the induction of a resolution phase is mandatory to prevent persistent harmful inflammation and oxidative stress in the host cells[76]; thus, the activation of the anti-inflammatory response is needed to minimize such side-effects[78]. Therefore, the leukocyte activation together with the up-regulation of a repertoire of anti-inflammatory cytokines (i.e. *il7, il6r, il20ra* and *il21r*) is representative of this intimate coordination between both processes, suggesting also an added anti-inflammatory response triggered by the dietary EOs. The modulation of cytokine expression and immune cell stimulation are the mechanisms attributed to the biological activity of garlic compounds[79]. In addition, there are also processes associated to the anti-inflammatory bioactivity of carvacrol and thymol[80,81]. For instance, carvacrol was described as an inhibitor of human neutrophil elastase[82]. In our study, the Serpin Family B Member 1 (*serpinb1*), a protein that inhibits neutrophil elastases protecting tissue from damage at inflammatory sites, was also up-regulated by dietary EOs, corroborating its anti-inflammatory properties. In fish, the up-regulation of inflammatory cytokine genes was observed in the gut of tilapia fed dietary garlic powder (1.0%)[17]. In juvenile gilthead seabream, the dietary inclusion of a commercial encapsulated combination of carvacrol and thymol EOs (0.01%) resulted in an enhancement of the intestine absorptive capacity that was attributed to the induction of anti-inflammatory and anti-proliferative gene markers[12]. The same commercial additive also demonstrated an immunostimulatory effect in juvenile hybrid tilapia[28].

Gills of gilthead seabream infested with *S. chrysophrii* showed an over-expression of apoptosis, cell proliferation and inflammation processes[44]. Thus, under an infective process, the combination of the pro- and anti-inflammatory mechanisms is also required for a successful pathogen eradication[78]. Although our transcriptomic profiling of gills was conducted at the end of the nutritional trial before fish being exposed to the ectoparasite, the build-up of a local former anti-inflammatory response induced by dietary EOs might delay the effect of the inflammatory outcome associated to *S. chrysophrii*; thus, potentially reducing tissue damage. This regulation observed in our study could enhance tissue protection and regeneration mechanisms involved in gill's responses against ectoparasites, not only by the stimulation of an inflammatory response, but also by means of antioxidant and anti-inflammatory processes.

One of the common characteristics of mucosal-associated lymphoid tissues, including the GIALT, is the presence of mucus-secreting cells[42]. The main components of mucus are mucins, high molecular weight glycoproteins (GPs) with numerous carbohydrate chains *O*-glycosidically linked to a protein core. Both commensal and pathogenic microorganisms[83–85], and likewise monogenean parasites[86,87], use this mucosal GPs as receptors for their attachment. A high variety of mucin oligosaccharides forms an extensive repertoire of attachment sites with different carbohydrate specificities[88]. Peculiarly, sialic acids and related saccharide residues can serve as receptor sites for binding exogenous macromolecules such as those of bacterial, viral or parasitical aetiology, playing an important role as "decoy" for pathogens, in such a strategy where the sialylated mucins are shed with the anchored sialic-binding pathogen[89,90]. Under parasite infections, qualitative changes of fish mucus occur, mainly in the mucin glycosylation pattern[91–93]. Thus, increases in sialic acid and *N*-acetylglucosamine terminal in mucins are described in as a host defence against helminths[94]. Nevertheless, many pathogens and parasites have evolved to disrupt the mucin barrier; for instance, several digestive mucins were down-regulated and a significant reduction of MC positive for sialic acid was observed in gut-parasitized gilthead seabream[95–97].

In our study, it is relevant to highlight an increase of carboxylated and/or sulphated GPs containing sialic acid and of *N*-acetylglucosamine/β-D-GlcNAc residues in gills of fish fed dietary EOs. Neutral GPs lubricate, facilitate gas exchange and regulate the acidity of mucous secretions, whereas acid carboxylated and sulphated GPs are more viscous, a characteristic associated to their antibacterial and antiparasitic properties[85,98–100]. The higher presence of acid GPs coupled with the increase in sialic acid and *N*-acetylglucosamine in mucins may be associated to an enhanced protection against *S. chrysophrii* attachment. Furthermore, MCs hypertrophy observed in gills of fish fed dietary EOs indicated a potentiation of the mucosal secretion and renewal, boosting its protective function. These results may be a consequence of the synergy between garlic, carvacrol and thymol EOs, since such histochemical differences and antiparasitic effects were not observed when garlic was tested by separate (Supplementary Information 1). Transcriptomics revealed that ALG9 Alpha-1,2-mannosyltraferase (*dibd1*), which is involved in *N*-glycan biosynthesis[101], was positively regulated by dietary EOs, which is one of the most common post-translational modifications of proteins[102]. Additionally, we found an up-regulation of *O*-Sialoglycoprotein Endopeptidase like 1 (*osgepl1*), whose enzyme is for long commercially used and recognized for its mucin-degrading activity, and which increase might indicate an enhance of the proteolytic mucin degradation[103], which is characteristic of the host GPs "shedding" defence mechanism. In this way, although not evidenced among the main representative processes in our gill's transcripteractome outcome, the regulation of some of pathways such as translational elongation, peptide biosynthesis, cellular protein metabolism, intracellular protein transport, and exocytosis-related processes, might be correlated with those of the mucosal surface.

Altogether, transcriptomic results suggest that dietary EOs may be promoting the synthesis and release of GPs detected at histological level, which might have a beneficial functionality on the gills and potentially reducing the *S. chrysophrii* attachment to gill's surface. Accordingly, gilthead seabream fed dietary EOs showed a reduction of 78% of total parasite load when compared with the control group, with a decrease in the prevalence of most of the parasitic developmental stages as well. Garlic is known for its wide-spectrum of antimicrobial activity that is attributed to allicin and ajoene, which exert multiple inhibitory effects on thiol-dependent enzymatic systems[104]. Similarly, garlic-based treatments demonstrated to be particularly effective in the fight against monogeneans and other parasites[18,105,106]. Farmed barramundi fed diets containing a garlic extract (50 and 150 mL kg^{-1}) for 30 days showed a reduction of *Neobenedenia* sp. oncomiracidia stage[19]. Similarly, garlic extract administered by balneation (0.76 and 15.2 µL L^{-1} allicin concentration) had also antiparasitic properties towards *Neobenedenia* sp.[22]. In guppy, both diet (10 and 20% garlic powder) and bath (7.5 and 12.5 mL L^{-1} garlic extract; 1 g L^{-1} fresh

crushed garlic) garlic-based treatments were successful against *Gyrodactylus turnbulli*[20]. The application of garlic bath treatments (3 ppt garlic oil; 300 mg L^{-1} crushed garlic cloves) resulted effective against *Trichodina* sp. and *Gyrodactylus* sp. in Nile tilapia[16]. However, an in vitro treatment with a water–ethanol extract of garlic tested at different dilutions (1:10, 1:50 and 1:100) showed no overall antiparasitic effect on *Neobenedenia* sp.[107]. The instability of free organosulfurs may lead to contradictory results in terms of the efficiency of garlic extracts and doses against monogenean parasites. These results highlight the benefits of encapsulating this type of compounds for dietary administration, since this process ensures their dietary stability, preventing inopportune interactions with the host and environment, and allowing their proper delivery in the gastrointestinal tract[4].

Concerning carvacrol and thymol, several bath treatments with different EOs proved to be effective anthelmintics[29–31]. The antiparasitic action of carvacrol against protozoans in chum salmon was associated to its presence in the skin of fish fed a diet supplemented with oregano EO at 0.02%[109]. Thymol was also demonstrated to have antiparasitic effects against the protozoan *Leishmania* sp.[108] and sheep gastrointestinal nematode helminths[109]. Nonetheless, there are few studies that accurately describe the antiparasitic effect of dietary phytochemicals against monogeneans in fish species; thus, the pathways and mechanisms of their action are not clear yet[110]. Regardless of this fact, present transcriptomic data from gills at the end of the nutritional trial provided the base line knowledge for deciphering the antiparasitic role of the tested EOs. While fish immunity against monogenean parasites is certainly multifactorial and innate factors seem to dominate the first response against this parasites[111–113], the participation of the mucosal adaptive factors, such as B-lymphocytes[42,114], immunoglobulins[115–117] or even specific antibodies[118–120], could be critical for longstanding parasite suppression[71,86]. Responses against helminth parasites also include the expression of classical effector type 2 cytokines that will signal the recruitment of inflammatory cells and induce goblet cell hyperplasia leading to mucus production[121]. Nevertheless, the specific mechanisms modulated during the parasitic challenge were not evaluated in this work, thus further studies are needed to determine if a type 2 immune response is also implicated in the success of the diet-induced antiparasitic response. Studies focused on identifying the bioactive compounds responsible for the gill's response observed in the present work are currently in progress.

Concluding, we showed that the administration of a microencapsulated feed additive containing garlic, carvacrol and thymol EOs in gilthead seabream promoted the activation of protein biosynthetic processes in gills. These biogenic processes are highly related to the translation of mRNA into proteins, which in turn are actively mobilized by vesicular transport and exocytosis. This mechanism activates effector leukocytes like acidophilic granulocytes. The immune response promoted by dietary EOs is also supported by the active control of oxidation–reduction processes, the building of an anti-inflammatory local response and the changes in the histochemical properties of mucins produced by branchial MCs. The overall results of our study highlighted the main biological processes induced by this dietary EOs that might be responsible for the later antiparasitic response observed in gills against *S. chrysophrii*. The notorious effect of the tested dietary EOs suggests its application as preventive and active treatment for this particular ectoparasite and a promising alternative treatment for other infections, although further evaluation is needed in order to validate this hypothesis.

Methods

Diets. A basal diet (46% crude protein; 18% crude fat; energy: 21.5 MJ kg^{-1}) was formulated (Table 3) to meet the nutritional requirements of gilthead seabream under summer conditions[40]. The experimental diet contained a microencapsulated additive at 0.5% composed of a blend of garlic, carvacrol and thymol synthetic EOs (AROTEC-G, TECNOVIT-FARMFAES S.L., Spain). Both extruded diets (pellet size: 2 mm) were manufactured by SPAROS Lda. (Portugal).

Fish rearing and nutritional assay. Gilthead seabream (body weight, BW = 5.0 ± 0.2 g; mean ± standard deviation) were obtained from Piscicultura Marina Mediterránea SL (Spain). After 105 days, 150 fish (BW = 40.3 ± 0.1 g) were distributed in six 450 L tanks connected to an IRTAmar recirculation system under open-flow water regimen and natural photoperiod (geographical coordinates ETRS89 system = 0.660418 E, 40.627516 N). Water temperature (24.6 ± 1.6 °C; range 21–28 °C; Fig. 10), oxygen (7.0 ± 1.7 mg L^{-1}; > 80% saturation) (OXI330, Crison Instruments) and pH (7.5 ± 0.01) (pHmeter 507, Crison Instruments) were daily controlled. Salinity (35‰) (MASTER-20 T; ATAGO Co. Ltd), ammonia (0.13 ± 0.1 mg NH_4^+ L^{-1}) and nitrite (0.18 ± 0.1 mg NO_2^- L^{-1}) levels (HACH-DR9000 Colorimeter, Hach) were weekly monitored. Data on water temperature and oxygen levels during the full experiment are depicted in Fig. 10.

The nutritional trial was run in triplicate (initial density = 2 kg m^{-3}; 25 fish $tank^{-1}$) during the summer and early autumn period. During 104 days, fish were fed both diets twice per day at apparent satiation (feeding rate = 3.0% of the stocked biomass). Fish were individually weighed at the beginning (end of July) and at the end of the nutritional assay (65 days, end of September), and at the end of the cohabitation/challenge period (104 days, end of October; see "Cohabitation challenge with *S. chrysophrii*"). At 65 days, four fish were selected from each tank, euthanized (MS-222, Sigma-Aldrich) and their second gill arch (right side) sampled for histological and transcriptomic analyses.

Transcriptional analysis. *RNA isolation and quality control.* Gills were fixed in RNAlater (Invitrogen, Thermo Fisher Scientific), incubated overnight (4 °C), and stored at − 80 °C. Approximately 20 mg of whole filaments of the gill lamellae medial portion were removed from the bone (~ 1 cm longitudinally close to the bone) and homogenized with a cell disrupter. Total RNA was extracted (n = 8 fish per diet) using the RNeasy Mini Kit (Qiagen, Germany) and eluted (final volume = 35 µL) in nuclease-free water and treated with DNAse (DNA-free DNA Removal Kit; Invitrogen). Total RNA concentration and purity were quantified using a Nanodrop-2000 (Thermo Scientific) and stored at − 80 °C. Prior to hybridization, samples were diluted to 133.33 ng μL^{-1} and

Ingredients	Basal diet (%)
Fishmeal 70 LT FF Skagen	20.0
Fishmeal CORPESCA Super Prime	10.0
CPSP 90	2.5
Squid meal	2.5
Soy protein concentrate (Soycomil)	5.0
Wheat Gluten	5.0
Corn gluten	8.0
Korfeed 60	4.5
Soybean meal 48	8.0
Rapeseed meal	4.0
Sunflower meal	3.0
Wheat meal	7.0
Whole peas	2.5
Fish oil—COPPENS	9.0
Soybean oil	1.5
Rapeseed oil	2.5
Vitamin and mineral Premix PV01	2.0
Soy lecithin—Powder	2.0
Antioxidant powder (Paramega)	0.4
Dicalcium phosphate	0.6
TOTAL	100.0
Proximate composition, % in dry basis	
Crude protein	46.2
Crude fat	18.4
Gross energy	21.5

Table 3. Formulation and proximate composition of the basal diet used during the nutritional assay.

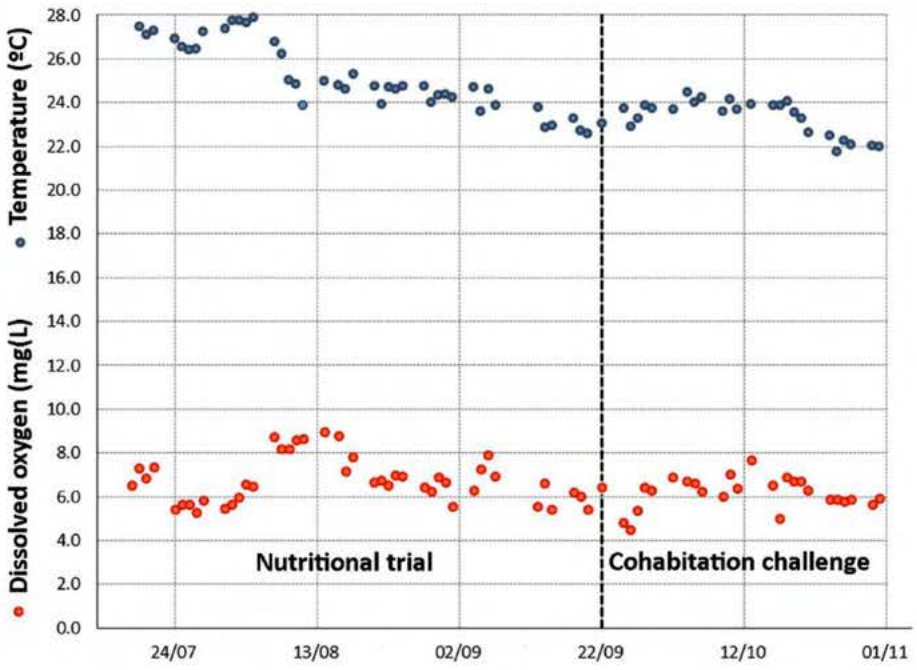


Figure 10. Daily mean values for water temperature (°C) and dissolved oxygen (mg L^{-1}) in experimental tanks along the nutritional and cohabitation trial conducted in order to evaluate the effect of a diet supplemented with a blend of essential oils (garlic, carvacrol and thymol) against an ectoparasite infestation by *Sparicotyle chrisophrii*. Daily data are computed using the individual values for each experimental tank (n = 6); no data for weekends or holidays are reported since these variables were not manually measured during these days.

checked for integrity (Agilent 2100 Bioanalyzer; Agilent Technologies, Spain). RNA samples (RIN value > 8.5) were pooled in three sets per diet (two sample pools with n = 4 fish each; and a third pool combining 1:1 of the former pools).

Microarray design, hybridization and analysis. Transcriptional analysis was done using the Aquagenomics *Sparus aurata* oligonucleotide microarray v2.0 (4 × 44 K) (SAQ) platform. Platform information and transcriptomic raw data are available through Gene Expression Omnibus (GEO) at NCBI (accession numbers GPL13442 and GSE144055, respectively).

Analyses were conducted using a one-color RNA labelling (Agilent One-Color RNA Spike-In kit; Agilent Technologies). Total RNA (200 ng) from each sample pool were reverse-transcribed with spike-in. Total RNA was used as template for Cyanine-3 labelled cRNA synthesis and amplification kit (Quick Amp Labelling kit). cRNA samples were purified using the RNeasy micro kit (Qiagen). Dye incorporation and cRNA yield were checked (NanoDrop ND-2000); Cy3-labeled cRNA (1.5 mg) with specific activity > 6.0 pmol Cy3/mg cRNA were fragmented (60 °C, 30 min), and then mixed with the hybridization buffer (Gene Expression Hybridization kit, Agilent Technologies), and hybridized (65 °C, 17 h) to the array (ID 025603, Agilent Technologies). Washes were conducted using Gene expression wash buffers, stabilization and drying solutions. Microarray slides were scanned (Agilent G2505B Microarray Scanner System) and spot intensities and other quality control features extracted (Agilent Feature Extraction software version 10.4.0.0).

Transcripteractome. The complete map of interactions (interactome) was obtained from differentially expressed genes (DEGs) obtained in the microarrays-based transcriptomic analysis, the so-called transcripteractome[122]. The Search Tool for the Retrieval of Interacting Genes (STRING) (https://string-db.org) was used[123]. Protein–protein interaction (PPI) network of DEGs was conducted with a high-confidence interaction score (0.9) using *Homo sapiens* dataset. Genecards[124] and Uniprot[125] databases were used to confirm match of gene acronym between *H. sapiens* and gilthead seabream. Gene ontology (GO) enrichment analysis for DEGs was performed with STRING ($P < 0.05$).

Histochemistry of the branchial tissue and mucous cells. The second gill arch from the right side was dissected from four fish per tank (n = 12 per diet) and fixed in 10% neutral-buffered formalin. After dehydration, tissues were embedded in paraffin and sectioned (3–5 µm thick). Two sections were stained with haematoxylin–eosin; the rest were used for evaluating the histochemical properties of branchial epithelia and mucous cells. These histochemical techniques were performed: Schiff, Periodic Acid Schiff (PAS), diastase-PAS, KOH-PAS, Alcian Blue (AB) pH 0.5, 1.0 and 2.5, and neuraminidase-AB pH 2.5 Underwood[126]. For characterization of glucidic residues bound to glycoconjugates, these horseradish peroxidase (HRP) conjugated lectins (Sigma-Aldrich, Spain) were used: *Canavalia ensiformes*/ConA (Mannose and/or Glucose), *Triticum vulgaris*/WGA (*N*-acetyl-D-glucosamine and/or *N*-acetylneuraminic acid, NeuNAc/sialic acid/NANA), *Ulex europeus*/UEA-I (L-Fucose), *Sambucus nigra*/SNA (NeuNAc/sialic acid/NANA) and *Glycine max*/SBA (α-*N*-acetyl-D-galactosamine). Sections were treated with 0.3% H_2O_2 for 10 min (endogenous peroxidase inhibition) in Tris-buffered saline (TBS; pH 7.2) and incubated for 30 min at RT in HPR-lectin conjugated (20 µg mL^{-1}) dissolved in TBS. After three TBS washes, peroxidase activity was visualized with TBS containing 0.05% 3,3′-diaminobenzidine tetrahydrochloride and 0.015% H_2O_2. Sections were washed in running water (10 min), dehydrated, cleared and mounted. Controls were described as in Sarasquete, et al.[84]. Histochemical results were visualized under a light microscope (Leitz diaplan) and manually registered on a table. Results were expressed as the semiquantitative assessment of colour intensity scores [0, negative; 1, weak; 2, moderate; 3, intense; 4, very intense] from four independent observers (Supplementary Information 2). Mucous cell count was determined in four different gill regions and their number expressed per length unit of the basal lamina (1 mm), according to Yamamoto et al.[127]. The size of mucous cells was measured as the surface area (expressed as μm^2) of 20 randomly selected cells of each gill section using a Spot (5.2) imaging software.

Cohabitation challenge with *S. chrysophrii*. *Establishment of a fish donors' stock.* Parasitized fish were obtained from sea cages of a private fish farm (data not provided for confidentiality purposes) and transported to IRTA facilities. Then, part of the parasitized fish were sacrificed, gills dissected and *S. chrysophrii* (juveniles and adults) placed in petri dishes with sea water until their inoculation in healthy fish. This strategy was chosen to avoid the presence of other branchial parasites[128]. Naïve fish previously anesthetized (MS222, 20 ppm) were infested on the left branchial lamellae with *S. chrysophrii* (n = 10 parasites $fish^{-1}$) with a Pasteur pipette. The presence of the parasite in gill lamellae was visually checked to confirm its successful attachment. In case the parasite did not properly attach to the gill lamella, it was rescued from the water and the infestive process repeated. Fish successfully infested were selected as "Trojan fish" for the cohabitation challenge, transferred to a quarantine tank and periodically sacrificed to confirm and estimate the number of parasites. A graphical summary of this process is presented in Fig. 11.

Cohabitation trial and parasite counting. The potential beneficial effect of the blend of EOs in infested fish was tested in a cohabitation challenge with *S. chrysophrii* (Fig. 11). For this purpose, 27 fish from each nutritional group (9 fish from each replicate tank; naïve fish) were randomly selected and moved to 450 L-tanks (n = 27 fish per tank; 1 tank per each diet administered). Each individual fish was considered as an experimental unit to meet the 3Rs principles of animal experimentation[129]. Therefore, welfare issues were assessed in agreement with good culture practices, where population density was established within each tank without jeopardizing the infestation procedure and its efficiency. Trojan (infested) fish (BW = 110.5 ± 6.6 g) were randomly selected

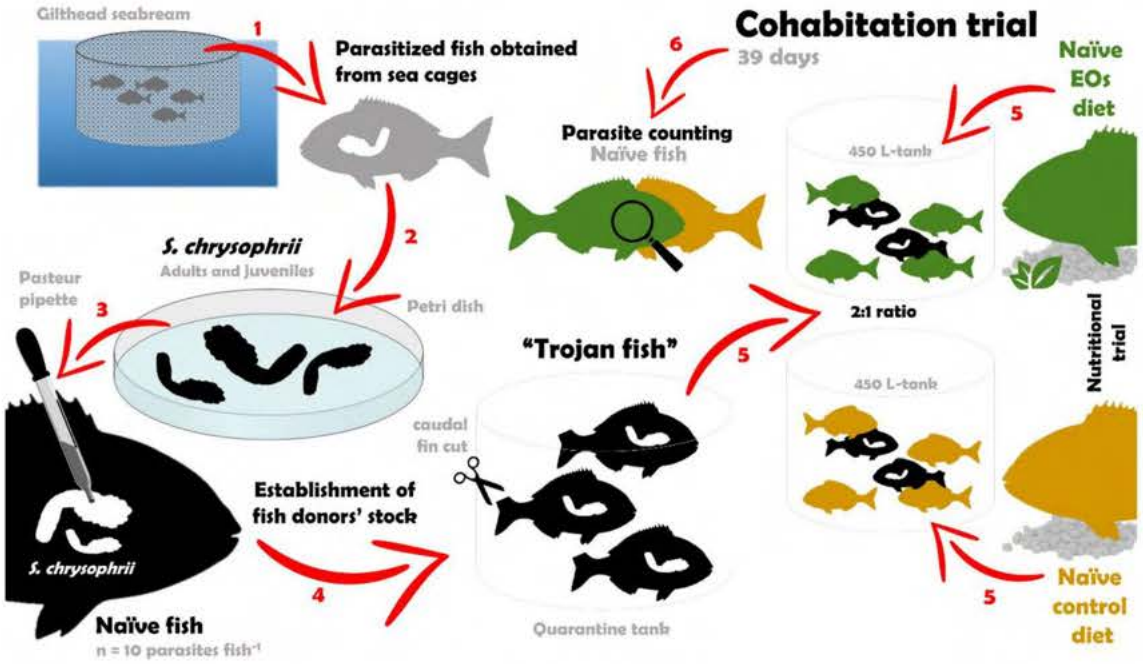


Figure 11. Schematic representation of the cohabitation trial set-up (see "Methods" section for details of each step).

from the parasitized fish tank and the tip of their caudal fin cut to distinguish them from naïve ones. Given the low infestation rate on the Trojan fish, a 2:1 ratio (27 naïve: 14 Trojans) was used. Each tank (naïve and Trojan) was fed the same diet (control and EOs diets). The cohabitation trial lasted 39 days (104 days from the beginning of the nutritional trial), considering that a minimum of 3–5 weeks is needed to successfully parasitize naïve fish under a cohabitation challenge model[130]. At the end of the challenge, naïve fish from each of the tanks (n = 15) were randomly weighed, sacrificed with an overdose of anesthetic and frozen until parasite counting. The presence of parasites was checked in all branchial arches (right and left), counted one by one in each gill filament using a stereomicroscope, and classified as adults, juveniles, post-larvae and eggs. The classification was attributed depending on the size and the number of clamps: post-larvae (small size—around 200 microns; 4–5 pairs of clamps), juveniles (medium size—around 2000 microns; 20–30 pairs of clamps) and adults (long size—5000–6000 microns; around 50–60 pairs of clamps). Prevalence, intensity and abundance were calculated according to Rózsa et al.[131].

Animal experimental procedures were conducted in compliance with the research protocol approved by the IRTA's Committee of Ethics and Animal Experimentation and in accordance with the Guidelines of the European Union Council (86/609/EU) for the use of laboratory animals.

Statistics. Differences between BW were analysed with an unpaired t-test and each time point was analysed individually assuming data homoscedasticity (GraphPad PRISM 7.00). Microarrays extracted raw data were analysed with Genespring version 14.5 GX (Agilent Technologies). The 75% percentile normalization was used to standardize arrays for comparisons and data were filtered by expression. An unpaired t-test was conducted without correction to identify those DEGs between both diets. Principal component analysis (PCA), Venn diagram, and the hierarchical heatmap were obtained with Genespring version 14.5 GX. Mucous cell density and size were compared with a t-test analysis (SPSS, version 2.4). Differences in parasite number were compared by means of a Two-way ANOVA considering diets and parasite stages as independent factors. Statistical differences were set at P value < 0.05.

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Author contributions

A.E. and E.G. designed and carried out the experiments. Biological samplings were performed by E.G., A.E. and J.F. The transcriptomic analysis was performed by F.E.R.-L., E.V.V. and J.F.; J.B. and J.F. contributed to the transcriptomic results graphic design. C.S. and J.O.D. carried out the histochemical analyses. J.F. wrote the original draft. All the authors provided critical feedback and approved the final manuscript. The study was supervised by E.G., F.E.R.-L and L.T.

Competing interests

Joana P. Firmino is a current TECNOVIT-FARMFAES S.L employer conducting an Industrial PhD. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Additional information

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CHAPTER II

Diet, immunity and microbiota interactions: An integrative analysis of the intestine transcriptional response and microbiota modulation in gilthead seabream (*Sparus aurata*) fed an essential oils-based functional diet

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Diet, Immunity, and Microbiota Interactions: An Integrative Analysis of the Intestine Transcriptional Response and Microbiota Modulation in Gilthead Seabream (*Sparus aurata*) Fed an Essential Oils-Based Functional Diet

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Essential oils (EOs) are promising alternatives to chemotherapeutics in animal production due to their immunostimulant, antimicrobial, and antioxidant properties, without associated environmental or hazardous side effects. In the present study, the modulation of the transcriptional immune response (microarray analysis) and microbiota [16S Ribosomal RNA (rRNA) sequencing] in the intestine of the euryhaline fish gilthead seabream (*Sparus aurata*) fed a dietary supplementation of garlic, carvacrol, and thymol EOs was evaluated. The transcriptomic functional analysis showed the regulation of genes related to processes of proteolysis and inflammatory modulation, immunity, transport and secretion, response to cyclic compounds, symbiosis, and RNA metabolism in fish fed the EOs-supplemented diet. Particularly, the activation of leukocytes, such as acidophilic granulocytes, was suggested to be the primary actors of the innate immune response promoted by the tested functional feed additive in the gut. Fish growth performance and gut microbiota alpha diversity indices were not affected, while dietary EOs promoted alterations in bacterial abundances in terms of phylum, class, and genus. Subtle, but significant alterations in microbiota composition, such as the decrease in *Bacteroidia* and *Clostridia* classes, were suggested to participate in the modulation of the intestine transcriptional immune profile observed in fish fed the EOs diet. Moreover, regarding microbiota functionality, increased bacterial sequences associated with glutathione and lipid metabolisms, among others, detected in fish fed the EOs supported the metabolic alterations suggested to potentially affect the observed

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immune-related transcriptional response. The overall results indicated that the tested dietary EOs may promote intestinal local immunity through the impact of the EOs on the host-microbial co-metabolism and consequent regulation of significant biological processes, evidencing the crosstalk between gut and microbiota in the inflammatory regulation upon administration of immunostimulant feed additives.

Keywords: gut-associated lymphoid tissue, microbiota, additive, functional feed, aquaculture, oral immmunization, teleost, gut immune crosstalk

INTRODUCTION

In the post-antibiotics era, concerns about the potential loss of productivity due to an increase of infectious diseases are a reality within the animal production sector. The production of aquatic protein through aquaculture is not an exception. Regarding the aquaculture industry, the increasing pathogen resistance to chemotherapeutic treatments and their use restrictions, along with the rising public awareness regarding food safety, environmental impact, and animal welfare, have encouraged the development of alternative dietary treatments, such as functional feeds (1). Similarly, to livestock, functional feed additives may benefit farmed fish through the regulation of the host metabolism, nutrient absorption, and enhancement of host performance. Furthermore, it may activate the immunity of the host either by direct stimulation of the innate immune system or through the sustenance of commensal microorganisms and inhibition of pathogens in the intestinal tract (2). These factors, either individually or combined, may have a profound impact on key performance indicators. For instance, fish farmers recognize the direct effect of health promotion through feed in their economic gain by the improvement of key performance indicators (i.e., improving somatic growth, reducing feed conversion rates, promoting host welfare, and diminishing morbidity, among others). The wide array of potential benefits derived from functional feed additives and nutraceuticals has focused the light on their study and validation in aquafeeds. Among functional feed additives, phytogenics consist of a heterogeneous group of plant-derived products widely used in animal nutrition. Essential oils (EOs), a blend of organic substances synthesized by aromatic plants during secondary metabolism, whose chemical composition may vary according to plant and environment characteristics and/or extraction procedures, are the most common class of phytogenics used in livestock nutrition as well as in aquafeeds. EOs have been increasingly studied as promising chemotherapeutic alternatives due to their antimicrobial, immunostimulant, antioxidant, anti-stress, and growth-promoting properties, without associated environmental or hazardous side effects. In addition, there is also evidence that EOs may exert a positive impact on the gut health of livestock, including aquaculture relevant fish species (3). Particularly, some studies have recently reported the advantageous outcomes of the dietary administration of garlic (4), carvacrol, and/or thymol (5) in the gut health of aquatic species, which suggest them as interesting phytogenic targets for aquafeed additive development.

During the last years, the concept of "gut health" has become a trending topic due to its significance on the nutrition, metabolism, immunity, pathogen control, welfare, behavior, and performance of the host. Despite the term not being well-defined in the literature and perhaps having a subjective definition, the key components of this term in animal production are: (i) the diet, (ii) the functional structure of the mucosal barrier, (iii) an effective digestion and absorption of nutrients, (iv) an appropriate and stable microbiota, and (v) an effective immunity (6). Similar to higher vertebrates, teleost fish have a specialized and sophisticated gut immune system, although a significant variation is observed in the gastrointestinal tract of different species according to their nutritional requirements (7). The intestinal mucosal layer (including the mucus and the epithelial cells) form an important physical and biochemical defense barrier against exogenous substances, such as bacteria, toxins, and allergens. It also participates in the local immune response through the recognition and processing of antigens, the recruitment of innate and adaptive immune cells, and the secretion of cytokines, chemokines, antimicrobial peptides, and mucins through the gut-associated lymphoid tissue (GALT) (2).

A key actor in gut integrity and functionality is the microbial community that colonizes it. In this sense, the gut commensal microbiota, besides protecting the host against pathogenic bacteria invasion, are able to modulate the gene expression of processes involved in the stimulation of epithelial proliferation, nutrient metabolism, and innate immune responses, promoting intestinal homeostasis (3). Under this context, any interference on the intestinal mucosal barrier integrity and/or the microbiota composition may impair gut condition and health, leading to disease-related dysbiosis (8). An increasing body of evidence supports the general assumption that feed additives can influence considerably the fish gut condition, affecting the intestinal epithelium, microbiota, and mucosal immunity (2). There is also evidence that fish innate and adaptive immune system may influence the regulation and composition of the gut microbiota and *vice versa*. Such interactions are not clearly deciphered so far, especially in lower vertebrates (8). In this way, functional genomics studies provide a wide range of applications that allows an increased and better understanding of the mechanisms underlying this symbiosis. Consequently, this information is of paramount importance for deciphering the mode of action of feed additives and their proper dietary administration.

Under this context, the present study aimed to evaluate the intestinal tissue transcriptional activity and microbiota responses in the gut of the euryhaline fish gilthead seabream (*Sparus aurata*)

fed a functional feed additive consisting of a microencapsulated blend of garlic, carvacrol, and thymol EOs. Additionally, the authors also sought to provide new insights about the shared role of host-microbial co-metabolism in building-up a local immune response promoted by the tested feed additive.

MATERIALS AND METHODS

Rearing Conditions

Gilthead seabream fry (body weight, BW = 5.0 ± 0.2 g; mean ± SD) were purchased from Piscicultura Marina Mediterránea S.L. (Andromeda Group, Valencia, Spain) and transported to the research facilities of the Institute of Agrifood Research and Technology (IRTA) in Sant Carles de La Ràpita (Tarragona, Spain). Fish were randomly distributed among six tanks (450 L capacity) connected to the IRTAmar® recirculation system (5–10% water replacement per day for compensating evaporation and siphoning losses) in order to keep water quality through UV, biological, and mechanical filtration. At the beginning of the trial, 150 juveniles (25 fish per tank; initial density = 2 kg m^{-3}) were individually measured in body weight (BW, g) and standard length (SL, mm) to the nearest 0.1 g and 1 mm, respectively (BW = 40.3 ± 0.1 g; SL = 12.0 ± 0.2 mm). This assay took place under natural photoperiod, with daily monitoring of the water temperature (25.1 ± 1.5°C), oxygen (6.8 ± 1.7 mg/L; >80% saturation) (OXI330, Crison Instruments, Barcelona, Spain), and pH (7.5 ± 0.01) (pHmeter 507, Crison Instruments), whereas salinity (35‰) (MASTER-20 T; ATAGO Co. Ltd), ammonia (0.13 ± 0.1 mg NH_4^+/L), and nitrite (0.18 ± 0.1 mg NO_2^-/L) levels (HACH DR9000 Colorimeter, Hach®, Spain) were weekly controlled.

Diets and Feeding Trial

Diets were manufactured by Sparos Lda. (Olhão, Portugal) as follows: main ingredients were ground (below 250 μm) in a micropulverizer hammer mill (SH1; Hosokawa Micron, B.V., Doetinchem, The Netherlands). Powder ingredients and oils were then mixed according to the target formulation in a paddle mixer (RM90; Mainca, S.L., Granollers, Spain). After extrusion (2 mm pellet size), all feed batches were dried in a convection oven (OP 750-UF; LTE Scientifics, Oldham, UK) for 4 h at 45°C.

A basal (control) diet was formulated with high levels of marine-derived protein sources to contain 46% crude protein, 18% crude fat, and 21.5 MJ/kg gross energy (**Table 1**) as described in Firmino et al. (9). The second experimental diet was the control diet supplemented with 0.5% of the functional additive composed of a blend of microencapsulated garlic, carvacrol, and thymol synthetic EOs (AROTEC-G®, TECNOVIT-FARMFAES, S.L., Spain). When dealing with feed additives, especially EOs, their proper and controlled administration is of special importance. Thus, encapsulation technology was used for EOs incorporation into the experimental diet in order to improve its bioavailability and efficacy, as well as the standardization of its dosing in order to avoid variability and discrepancies among studies (10).

Diets were tested for 65 days in a feeding trial carried out in triplicate tanks. Fish were hand-fed two times per day at the daily rate of 3.0% of the stocked biomass, which approached apparent satiation. At the end of the trial, all the fish in each tank were netted, anesthetized (buffered 150 mg/L MS-222, Sigma-Aldrich, Spain), and measured for BW and SL. We calculated different performance parameters: specific growth rate (SGR; % BW/day) = 100 × (ln BWf – ln BWi)/days (where BWf and BWi represented the final and the initial body weights, respectively). Fulton's condition factor (K) = (BWf/SLf^3) × 100 (where SLf was the final SL). This is a morphometric index that estimates the body condition of fish, assuming that heavier fish of a given length are in better condition (11).

In addition, four fish were randomly selected from each tank, euthanized with an overdose of the abovementioned anesthetic, and their gut removed. For transcriptional analysis purposes, a small section of the mid-anterior intestine from each fish was dissected, placed in RNAlater™ (Invitrogen, Thermo Fisher Scientific, Lithuania), incubated overnight (4°C), and stored at −80°C until further RNA extraction. There is evidence that the mid-anterior section of the fish intestine has a specialized immunological functionality when compared with other intestinal sections (12). The remaining sections of the anterior and posterior intestine were frozen separately in dry ice and stored at −80°C for further microbiota analysis.

Transcriptional Analysis

RNA Isolation and Quality Control

Gilthead seabream mid-anterior intestine samples were randomly selected per dietary treatment. Total RNA was extracted individually using the RNeasy® Mini Kit (Qiagen, Germany) and eluted (final volume = 35 μl) in nuclease-free water and treated with DNAse (DNA-free™ DNA Removal Kit; Invitrogen, Lithuania). Total RNA concentration and purity were quantified using a Nanodrop-2000® spectrophotometer (Thermo Scientific, USA) and stored at −80°C. Prior to hybridization with microarrays, RNA samples were checked for RNA integrity (Agilent 2100 Bioanalyzer; Agilent Technologies, Spain) and selected by the criteria of a RIN value >8.5. Three different pools of samples per dietary treatment were established ($n = 4$ fish each).

Microarrays

A transcriptional analysis for the intestine from both experimental groups was carried out using the Aquagenomics *S. aurata* oligonucleotide microarray v2.0 (4 × 44 K) (SAQ) platform. Detailed information and transcriptomic raw data are available at the Gene Expression Omnibus (GEO) public repository at the U.S. National Center for Biotechnology Information (NCBI), accession numbers GPL13442 and GSE159643, respectively. The sampling labeling, hybridization, washes, and scanning were performed as described before (9). For this purpose, a one-color RNA labeling was used (Agilent One-Color RNA Spike-In Kit; Agilent Technologies, USA). RNA from each sample pool (200 ng) was reverse-transcribed together with the RNA spike-in. Then, total RNA was used as a template for Cyanine-3 (Cy3) labeled cRNA synthesis and amplification with the Quick Amp Labeling Kit (Agilent Technologies). cRNA samples were purified using the RNeasy Micro Kit (Qiagen). Dye

TABLE 1 | Formulation and proximate composition of the basal diet.

Ingredients	Basal diet (%)
Fishmeal 70 LT FF Skagen	20.0
Fishmeal CORPESCA Super Prime	10.0
CPSP 90	2.5
Squid meal	2.5
Soy protein concentrate (Soycomil)	5.0
Wheat gluten	5.0
Corn gluten	8.0
Korfeed 60	4.5
Soybean meal 48	8.0
Rapeseed meal	4.0
Sunflower meal	3.0
Wheat meal	7.0
Whole peas	2.5
Fish oil—COPPENS	9.0
Soybean oil	1.5
Rapeseed oil	2.5
Vitamin and mineral Premix PV01	2.0
Soy lecithin—powder	2.0
Antioxidant powder (Paramega)	0.4
Dicalcium phosphate	0.6
TOTAL	100.0
Proximate composition, % in dry basis	
Crude protein	46.2
Crude fat	18.4
Gross Energy	21.5

incorporation and cRNA yield were checked (NanoDrop ND-2000® spectrophotometer). Then, Cy3-labeled cRNA (1.5 mg) with specific activity > 6.0 pmol Cy3/mg cRNA was fragmented at 60°C for 30 min, and hybridized with the array in presence of a hybridization buffer (Gene Expression Hybridization Kit, Agilent Technologies) at 65°C for 17 h. For washes, microarrays were incubated with Gene Expression wash buffers and stabilization and drying solution according to the manufacturer's instructions (Agilent Technologies). Microarray slides were then scanned (Agilent G2505B Microarray Scanner System), and spot intensities and other quality control features were extracted (Agilent Feature Extraction software version 10.4.0.0).

Intestine Functional Analysis: The Search Tool for the Retrieval of Interacting Genes

The Search Tool for the Retrieval of Interacting Genes (STRING) public repository version 11.0 (https://string-db.org) was used to generate the transcripteractome that takes place in the intestine of fish fed the EOs-supplemented diet. A Protein–Protein interaction (PPI) Networks Functional Enrichment Analysis for all the differentially expressed genes (DEGs) was conducted with a high-confidence interaction score (0.9) using *Homo sapiens* as model organism. Gene ontology (GO) enrichment analysis ($p < 0.05$) was also assessed including all the DEGs obtained. To confirm gene orthologs and match gene acronyms between both *H. sapiens* and gilthead seabream species, protein BLAST was run, and the GeneCards (www.genecards.org) and UniProt (www.uniprot.org) databases were accessed as described in Firmino et al. (9).

Intestinal Microbiota

DNA Extraction

Samples were thawed gradually on ice, and the intestinal contents were extracted by pressing toward the ends with a sterile object. After homogenizing the content, a sample (50 mg) was taken for DNA extraction following the protocol based on saline precipitation (13). DNA concentration was quantified fluorometrically with the Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) and its purity and integrity assessed using a NanoDrop™ One UV-Vis Spectrophotometer WiFi (Thermo Scientific, USA) and through an agarose gel electrophoresis.

Amplicon Sequencing of 16S Ribosomal RNA and Sequence Data Processing

The 16S rRNA of samples were sequenced on an Illumina® MiSeq platform (Illumina, San Diego, CA, USA) with 2 × 300 bp paired-end sequencing in the Ultrasequencing Service of the Bioinnovation Center of University of Málaga (Málaga, Spain). Sequencing was carried out using the sense forward 5′ TCGTCG GCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGG CWGCAG 3′ and 5′ GTCTCGTGGGCTCGGAGATGTGTAT AAGAGACAGGACTACHVGGGTATCTAATCC 3′ reverse primers directed to the variable regions V3–V4 of the 16S rRNA gene. All Illumina reads were analyzed using the FastaQC software in order to assess sequence quality. Further data processing including trimming and 16S rRNA analysis and visualization was performed with a workflow based on the mothur software package (1.39.5 version). Briefly, chimeras were detected using the software UCHIME version 4.2 (https://drive5.com/uchime, effective tags obtained) and sequences were aligned and clustered into operational taxonomic units (OTUs) with an identity cut of 80%. The total count threshold was set at 0.005% using the Greengenes database (13).

Microbial Functional Analysis: PICRUSt

Within the metagenomic study, the analysis of the most represented functions of the microbial community was conducted. For this purpose, PICRUSt (version 1.1.3) was used for comparing the predicted functional profiles from the anterior and posterior intestines of gilthead seabream fed both administered diets. PICRUSt is a bioinformatics software designed to predict the functional profile of a microbial community based on the study of the 16S rRNA. Reads from 12 samples (three samples from intestine sample and treatment) were filtered by rarefaction curves from 46,331 reads, and singletons were removed. A total of 45,844 sequences, which clustered into 103 OTUs identified in the Greengenes database, were used for additional bioinformatics analysis (**Supplementary Figure 1**). Sequencing data were introduced into the PICRUSt bioinformatics software, and samples were normalized to the number of copies of the 16S rRNA. Functional

metagenomes for each sample were predicted from the Kyoto Encyclopedia of Genes and Genomes (KEGG) catalog and collapsed to specified KEGG levels.

Statistics

Differences between biometrical parameters of both experimental dietary groups were analyzed through an unpaired *t*-test assuming data homoscedasticity (GraphPad PRISM 7.00; $p < 0.05$).

Raw data extracted from microarrays were imported and analyzed using GeneSpring version 14.5 GX software (Agilent Technologies). The 75th percentile normalization was used to standardize the arrays for comparisons, and data were filtered by expression. The DEGs were obtained from a gene-level differential expression analysis. An unpaired *t*-test was conducted without correction ($p < 0.05$) to identify DEGs between dietary treatments. The DEGs were grouped according to their fold-change value (FC, $p < 0.05$) and represented using the GraphPad PRISM software. The Principal Component Analysis (PCA) on conditions was carried out using GeneSpring software; four eigenvectors were calculated using a covariance matrix to describe the aggrupation of the control and EOs groups in a 3D plot. The gene expression values (log2-expression ratios) were represented by a hierarchical clustering heatmap analysis (MeV software v4.0), with Pearson distance and average linkage (9).

All data analysis of the intestinal microbiota was processed using Phyloseq and Vegan libraries in R statistical package. Readings were normalized based on rarefaction curves (46,331 reads) and singletons were removed. In addition, it was calculated the coverage using the Good's coverage coefficient, as well as the ecological indexes. Alpha diversity was estimated using the Chao1, Shannon, and Simpson indices, to assess taxonomic wealth, diversity, and dominance, respectively. For statistical analyses between diversity indices, the *t*-test ($p < 0.05$) was used; while the taxonomic comparison was carried out using the R package DESeq2 ($p < 0.01$). Differences in the functional prediction between diets were made using the ANOVA multiple comparison test with the Tukey-Kramer correction (corrected $p < 0.05$).

RESULTS

Growth Performance

At the end of the study, no significant differences were observed between fish fed the EOs-supplemented diet and the control diet in terms of somatic growth (BWf = 150.8 ± 14.9 vs. 157.8 ± 14.2 g; SLf = 17.1 ± 0.6 vs. 17.3 ± 0.6 mm), daily growth rates in terms of BW (SGR_{BW} = 2.03 ± 0.01 vs. 2.12 ± 0.07% BW/day) and Fulton's condition factor (K = 3.0 ± 0.1 vs. 3.1 ± 0.1), respectively (Unpaired *t*-test, $p > 0.05$). A survival rate of 96 and 92% was recorded for the EOs-supplemented and control diets, respectively.

Microarrays and Gut Transcripteractome

A total of 581 DEGs were found when comparing the transcriptomic profiling of the intestine from fish fed both diets ($p < 0.05$; **Supplementary Table 1**). The detailed analysis of gene FC revealed that genes were mostly upregulated in the fish fed the diet containing the functional additive (70.2% of DEGs), although its modulation was moderate in terms of FC intensity. In particular, 408 of the abovementioned DEGs were upregulated, with 404 of them within the $1.0 < FC < 1.5$ interval. The remaining four DEGs were grouped within the $2.0 \leq FC \leq 3.0$ interval. In addition, 173 DEGs were downregulated (29.8% of DEGs; $p < 0.05$) and all of them were grouped within the $-1.5 \leq FC \leq -1.0$ interval (**Figure 1A**). Common segregation among the pool samples within the same dietary treatment was observed in the hierarchical clustering of the intestine transcriptomic response based on correlation patterns from the DEGs response ($p < 0.05$) (**Figure 1B**). PCA analysis confirmed the differential transcriptomic profile among dietary treatments. The first component (Y-axis) accounted for 79.03% of variation; the second and third components (X-Axis and Z-axis, respectively) accounted for 6.26 and 6.03% of the variation; these three components show the perfect separation between the control diet and the fish fed with the additive (**Figure 1C**).

From the whole set of DEGs, a functional network analysis was performed. The transcripteractome showed 252 coding proteins (nodes) with 473 interactions (edges). The remaining 329 DEGs (annotated as unknown genes) were excluded from the analysis. Based on the 70 GO terms obtained from the enrichment analysis (**Supplementary Table 2**), 6 main representative groups for the biological processes were identified in the transcripteractome: (1) proteolysis; (2) immunity; (3) transport and secretion; (4) response to cyclic compounds; (5) symbiosis; and (6) gene expression (**Figure 2**).

The tested functional feed additive resulted in the positive regulation of biological processes related to the proteolysis category (32 upregulated genes and 16 downregulated genes; **Supplementary Figure 2**; **Supplementary Table 3**). Several biological processes were considered, namely, "proteolysis," "regulation of proteolysis," "regulation of peptidase activity," "regulation of endopeptidase activity," and "protein deubiquitination." In addition, biological processes associated with immunity showed a more balanced regulation in fish fed the EOs-supplemented diet (17 upregulated genes and 14 downregulated genes; **Supplementary Figure 3**; **Supplementary Table 4**). Several relevant GOs related to immunity were obtained, such as "cell activation," "leukocyte activation," "leukocyte activation involved in immune response," "neutrophil activation," "neutrophil degranulation," and "regulated exocytosis." The tested functional feed additive also favored biological processes associated with transport and secretion (50 upregulated genes and 26 downregulated genes; **Supplementary Figure 4**; **Supplementary Table 5**). Among them, "transport," "intracellular transport," "vesicle-mediated transport," and "secretion" processes were identified in the functional network. Moreover, some biological processes in the category of the response to cyclic compounds were positively affected by the dietary inclusion of EOs (25 upregulated genes and 11 downregulated genes; **Supplementary Figures 5, 6**; **Supplementary Table 6**). In particular, the following processes were evidenced, such as the "response to organic cyclic compound," "cellular response to organic cyclic compound,"

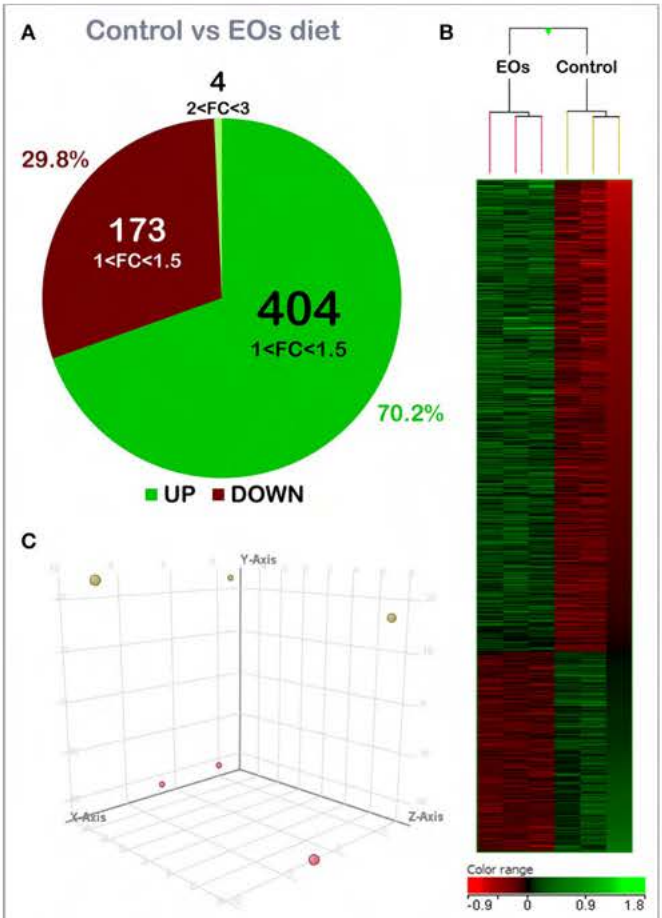

FIGURE 1 | Differential expression analysis of the gilthead seabream (*Sparus aurata*) mid-anterior intestine transcriptomic response to the garlic, carvacrol, and thymol essential oils (EOs)-supplemented diet. **(A)** Comparing both groups, 581 differentially expressed genes (DEGs) ($p < 0.05$) were found. From these, 408 genes were upregulated: 404 mainly concentrated in the 1.0–1.5-fold change (FC) interval; and 4 DEGs were grouped in the $2.0 \leq FC \leq 3.0$ interval. Additionally, 173 genes were downregulated ($p < 0.05$) and grouped in the range $-1.5 \leq FC \leq -1.0$. **(B)** Hierarchical clustering of the gilthead seabream mid-anterior intestine transcriptomic response for the control diet and EOs-supplemented diet, based on similitude patterns of the DEGs detected from three sample pools per dietary group. Data of the six microarrays are depicted, one for each represented pool. Both increased and decreased gene expression patterns are shown in green and red, respectively. All transcripts represented are statistically significant ($p < 0.05$). **(C)** Principal component analysis (PCA) of the DEGs of gilthead seabream intestine response to the control diet (yellow) and EOs-supplemented diet (red). PC1, PC2, and PC3 components loadings (Y-axis = component 1, 79.03% of variation; X-axis = component 2, 6.26% of variation; Z-axis = component 3, 6.03% variation). For DEGs details, please see also **Supplementary Table 1**.

"response to lipid," "cellular response to lipid," "response to hormone," "response to steroid hormone," "cellular response to hormone stimulus," "cellular response to steroid hormone stimulus," "response to alkaloid," "response to nitrogen compound," and "response to organonitrogen compound." Furthermore, symbiosis correlated biological processes, such as "symbiont process," "interspecies interaction between organisms," and "multi-organism process," were positively modulated (33 upregulated genes and 13 downregulated genes) in the intestine of fish fed the diet containing the functional feed additive (**Supplementary Figure 7**; **Supplementary Table 7**). Finally, the biological processes associated with gene expression and RNA processing (62 upregulated genes and 10 downregulated genes; **Supplementary Figure 8**; **Supplementary Table 8**), among them "gene expression," "RNA processing," "RNA splicing," "messenger RNA (mRNA) processing," "mRNA metabolic process," "mRNA export from nucleus," and "ribonucleoprotein complex export from nucleus" were observed to be much more upregulated in the intestine of fish fed the EOs-supplemented diet than in the control group.

A reasonable number of genes were observed to be shared among the immunity category and the other categories of gene expression/RNA processing (16%), proteolysis (29%), transport/secretion (77%), response to cyclic compounds (32%), and symbiosis (32%), suggesting a strong relationship between these biological processes that favors the host mucosal tissue immunity in response to the dietary EOs. Additionally, from the total DEGs obtained from the transcriptomic profile of the fish fed the EOs-supplemented diet, a set of genes was selected based on their expression and biological relevance (**Table 2**) in order to assess the participation of the intestine transcriptional regulation upon the observed microbiota modulation.

Intestine Microbiota Analysis

No variation was registered on the alpha diversity indices of the intestinal microbiota, regardless of the region of the intestine considered (**Table 3**; $p > 0.05$). However, the coefficient of variation (CV) for the Chao1 index was higher in the anterior (CV = 18.7 vs. 5.2%) and posterior (CV = 23.9 vs. 12.3%) intestinal segments of fish fed the diet containing the functional feed additive (**Supplementary Figure 9**). Similarly, the Shannon and Simpson diversity indexes were neither affected by the inclusion of the functional feed additive in the diet. Library coverage was calculated using Good's coverage index with a result of 99.98 ± 0.01.

The relative abundance of microbial taxa at the phylum level is shown in **Figure 3A**. Proteobacteria, Firmicutes, and Actinobacteria were commonly found in all samples regardless of the dietary condition and region of the intestine considered. However, only the phylum Spirochaetes showed significantly lower abundances in the posterior intestine in fish fed the diet supplemented with the functional feed additive ($p < 0.05$). At class level, *γ-Protebacteria* were the dominant group (60–73%; $p < 0.05$) in all samples assayed, whereas the abundance of *Clostridia* in the anterior intestine, and *Brevinematae* and *Bacteroidia* in the posterior intestine significantly decreased in fish fed the diet containing the blend of EOs (*t*-test, $p < 0.05$; **Figure 3B**).

At the genus taxonomic level, bacterial abundance (relative abundance > 1%) was significantly affected by the functional feed additive tested (*t*-test, $p < 0.05$). In particular, the anterior intestine of gilthead seabream fed the EOs-supplemented diet showed a significant increase (*t*-test, $p < 0.05$) in *Photobacterium* (γ-Proteobacteria, *Vibronaceae*) and *Corynebacterium*

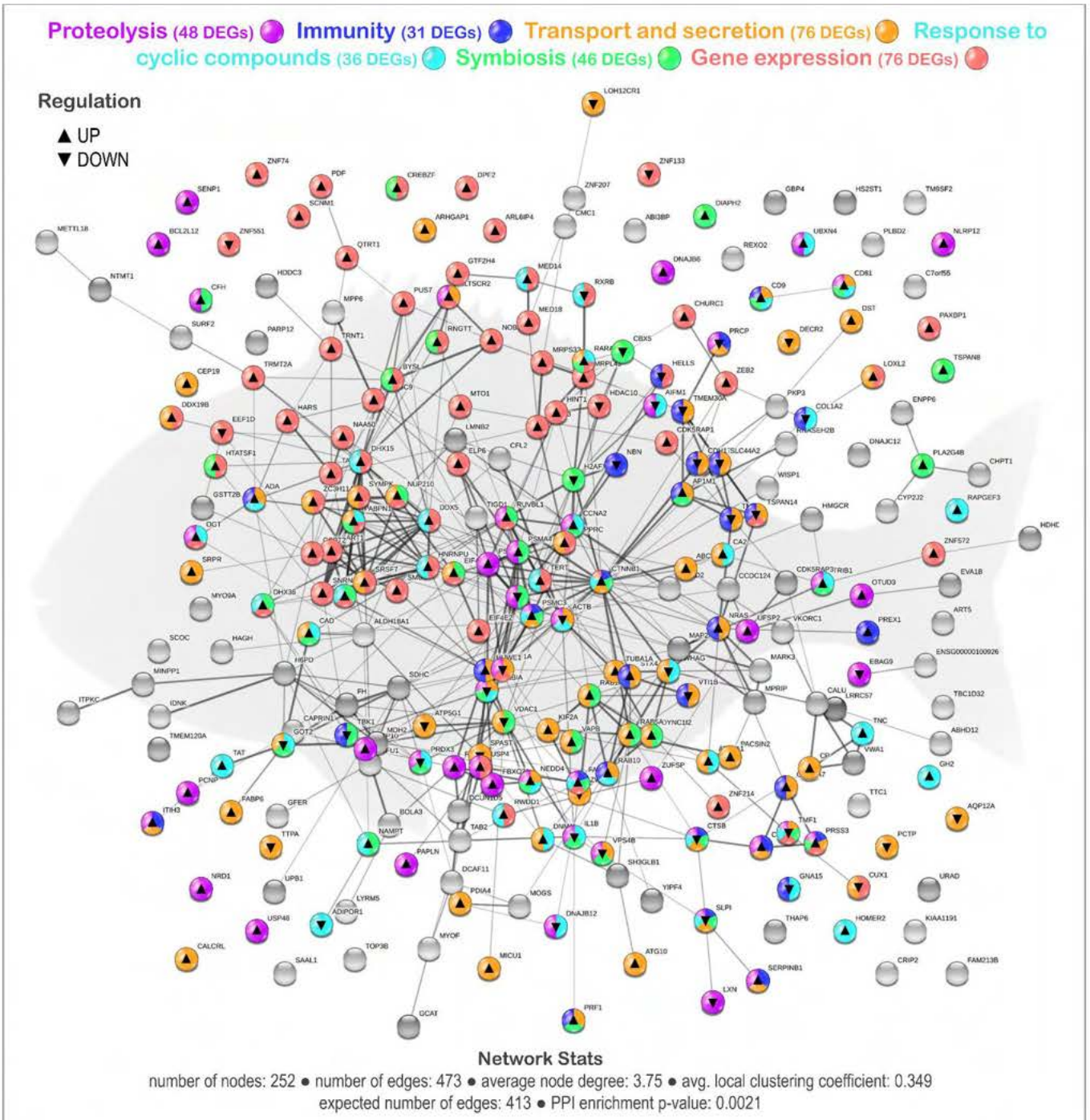

FIGURE 2 | Transcripteractome of the differentially expressed genes (DEGs) in the mid-anterior intestine of juvenile gilthead seabream (*S. aurata*) fed the garlic, carvacrol, and thymol essential oils (EOs)-supplemented diet (see also **Supplementary Tables** and **Supplementary Figures 2–8**). Protein–Protein Interactions Network (PPI) network nodes colors indicate the six representative processes identified from the functional enrichment analysis for each DEG represented—proteolysis, immunity, transport, and secretion, response to cyclic compounds, symbiosis, and gene expression. ▲ nodes represent upregulated genes and ▼ nodes represent downregulated genes. Graphic keys and network stats are indicated in the graphical figure legend.

(Actinobacteria, *Corynebacteriaceae*) abundance whereas a reduction in *Comamonas* (Proteobacteria, *Comamonadaceae*) was also found (*t*-test, $p < 0.05$; **Figure 4**). Regarding the posterior intestine, a significant decrease in the abundance of the genera *Paracoccus* (Proteobacteria, *Rhodobacteraceae*), *Prevotella* (Bacteroidetes, *Bacteroidaceae*), and *Rothia* (Actinobacteria, *Micrococcaceae*) was also detected in fish fed the functional feed additive (*t*-test, $p < 0.05$; **Figure 5**).

TABLE 2 | List of selected differentially expressed genes (DEGs) of the mid-anterior intestine of juvenile gilthead seabream (*Sparus aurata*) fed a diet supplemented with a blend of garlic, carvacrol, and thymol essential oils (EOs).

Gene name	Acronym	FC	*P*-value
PROTEOLYSIS AND DEUBIQUITINATION			
UFM1 Specific Peptidase 2	*ufsp2*	1.621	0.012
Proteasome 20S Subunit Alpha 6	*psma6*	1.491	0.014
Ubiquitin Specific Peptidase 10	*usp10*	1.490	0.026
Proteasome 26S Subunit, ATPase 3	*psmc3*	1.413	0.040
Ubiquitin Specific Peptidase 48	*usp48*	1.359	0.036
Proteasome 20S Subunit Alpha 4	*psma4*	1.289	0.027
OTU Deubiquitinase 3	*otud3*	1.256	0.035
Ubiquitin Specific Peptidase 4	*usp4*	1.103	0.007
Interleukin 1 Beta	*il-1β*	−1.167	0.025
NFKB Inhibitor Alpha	*nfkbia*	−1.327	0.047
Proteasome 20S Subunit Beta 6	*psmb6*	−1.495	0.048
IMMUNITY			
Leukocyte Elastase Inhibitor	*serpinb1*	1.561	0.024
Adenosine Deaminase	*ada*	1.517	0.025
Phosphatidylinositol-3,4,5-Trisphosphate Dependent Rac Exchange Factor 1	*prex1*	1.478	0.018
CD9 Molecule	*cd9*	1.283	0.033
CD81 Molecule	*cd81*	1.279	0.018
Perforin 1	*prf1*	1.181	0.034
Cathepsin B	*ctsb*	−1.340	0.031
TRANSPORT AND SECRETION			
Fatty Acid Binding Protein 6	*fabp6*	2.659	0.044
Serine Protease 3	*prss3*	1.824	0.002
RAB5A, Member RAS Oncogene Family	*rab5a*	1.668	0.023
RAB10, Member RAS Oncogene Family	*rab10*	1.456	0.030
Rho GTPase Activating Protein 1	*arhgap1*	1.363	0.048
NRAS Proto-Oncogene, GTPase	*nras*	1.257	0.010
RAB1A, Member RAS Oncogene Family	*rab1a*	1.238	0.025
Hypoxia Inducible Factor 1 Subunit Alpha	*hif1a*	−1.259	0.002
RESPONSE TO CYCLIC COMPOUNDS			
Carbonic Anhydrase 2	*ca2*	2.089	0.046
Tribbles Pseudokinase 1	*trib1*	1.740	0.011
Glutathione S-Transferase Theta 2B	*gstt2b*	1.456	0.018
Cytochrome P450 Family 2 Subfamily J Member 2	*cyp2j2*	1.284	0.045
Growth Hormone 2	*gh2*	1.283	0.032
ATPase Na+/K+ Transporting Subunit Alpha 1	*atp1a1*	1.271	0.022
Peroxiredoxin 3	*prdx3*	−1.220	0.009
Glutamic-Oxaloacetic Transaminase 2	*got2*	−1.254	0.008
Adiponectin Receptor 1	*adipor1*	−1.262	0.041
Cathepsin B	*ctsb*	−1.340	0.031
SYMBIOSIS			
Retinoic Acid Receptor Alpha	*rara*	1.164	0.003
Retinoid X Receptor Beta	*rxrb*	−1.172	0.030
GENE EXPRESSION			
Zinc Finger Protein 572	*znf572*	1.389	0.019
CDK5 Regulatory Subunit Associated Protein 3	*cdk5rap3*	1.341	0.014
Zinc Finger E-Box Binding Homeobox 2	*zeb2*	1.315	0.003
Heterogeneous Nuclear Ribonucleoprotein U	*hnrnpu*	1.284	0.012
Cellular Communication Network Factor 4	*wisp1*	1.280	0.040
NOP53 Ribosome Biogenesis Factor	*gltscr2*	1.263	0.034
Zinc Finger Protein 74	*znf74*	1.256	0.038
Pre-MRNA Processing Factor 8	*prpf8*	1.214	0.005
Zinc Finger CCCH-Type Containing 11A	*zc3h11a*	1.207	0.023
Zinc Finger Protein 214	*znf214*	1.201	0.008
Small Nuclear Ribonucleoprotein U5 Subunit 200	*snrnp200*	1.192	0.008
F-Box Protein 31	*fbxo31*	1.178	0.021
Spliceosome Associated Factor 1, Recruiter Of U4/U6.U5 Tri-SnRNP	*sart1*	1.155	0.005
Zinc Finger Protein 133	*znf133*	−1.127	0.006
Zinc Finger Protein 551	*znf551*	−1.168	0.001
Nibrin	*nbn*	−1.238	0.031

Genes were arranged according to six representative processes identified from the functional enrichment analysis. Gene description, respective acronym, fold-change intensity (FC), modulation (green: upregulation; red: downregulation), and p-value are described.

TABLE 3 | Alpha diversity of bacterial communities in the anterior and posterior intestinal tract sections of gilthead seabream (*Sparus aurata*) fed a control and the diet supplemented with a blend of garlic, carvacrol, and thymol essential oils (EOs).

			Chao1	Shannon	Simpson
Intestine	Anterior	Control	64.583 ± 3.357	2.513 ± 0.231	0.863 ± 0.046
		EOs diet	69.437 ± 13.013	2.288 ± 0.470	0.838 ± 0.093
	Posterior	Control	58.167 ± 7.182	2.401 ± 0.550	0.807 ± 0.164
		EOs diet	62.770 ± 14.983	2.363 ± 0.220	0.863 ± 0.039

The PICRUSt analysis enabled the prediction of the functional capacities of the microbial communities detected in the gilthead seabream intestine based on the treatment applied. The low Nearest Sequenced Taxon Index (NSTI) value (0.04 ± 0.02) from the PICRUSt analysis indicated a good prediction accuracy. The functional analysis of KEGG pathways revealed significant differences at second- and third-level classification KEGG pathways in both sections of the intestine (ANOVA, $p < 0.05$). Regarding the anterior intestine and considering the second-level classification of KEGG pathways, a reduction in carbohydrate metabolism in fish fed the diet supplemented with the functional feed additive was obtained (ANOVA, $p < 0.05$) (**Figure 6A**). When considering the third-level classification of KEGG pathways in the anterior intestine, a larger proportion

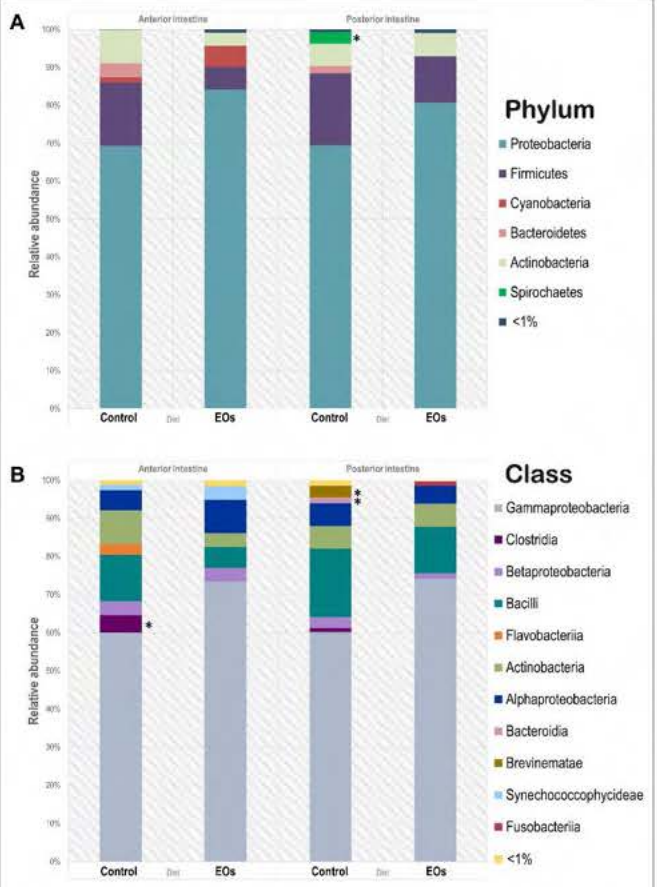

FIGURE 3 | Microbiota composition at phylum **(A)** and class **(B)** level of the anterior and posterior sections of the intestinal tract of gilthead seabream (*S. aurata*) fed control and garlic, carvacrol, and thymol essential oils (EOs)-supplemented diet. Asterisk (*) indicate significant differences among diets ($p > 0.05$).

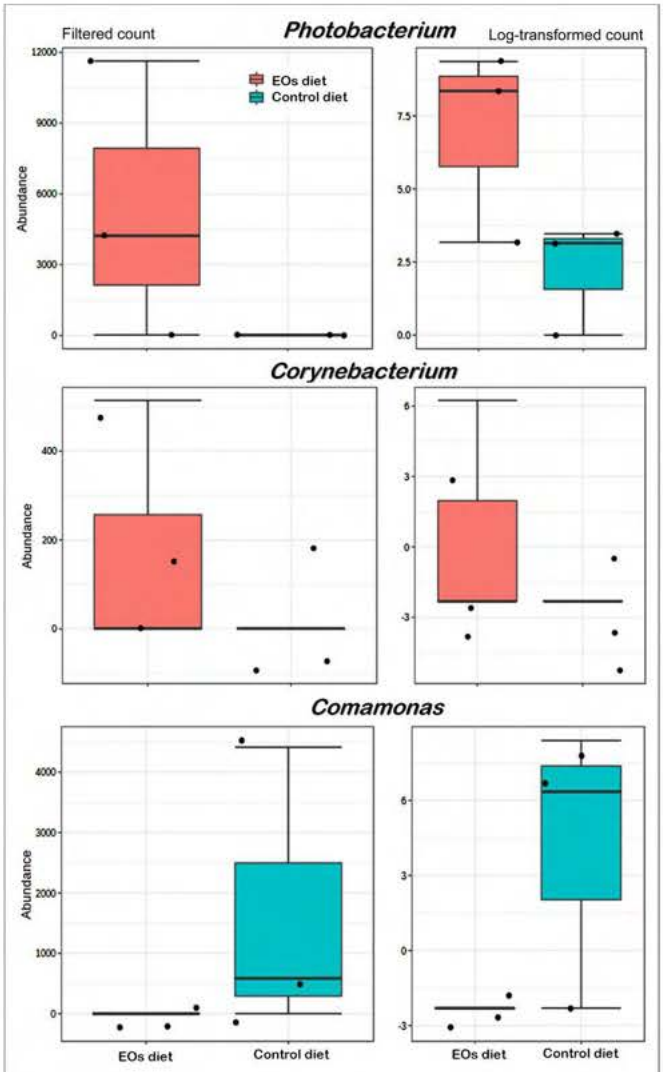

FIGURE 4 | Average abundances of genera showing significant differences ($p < 0.01$) in the anterior intestine of gilthead seabream (*S. aurata*) fed the garlic, carvacrol, and thymol essential oils (EOs)-supplemented diet in comparison with fish receiving the control diet.

of sequences associated with glutathione and lipid metabolism, and a reduction of sequences related to drug metabolism was found in fish fed the EOs-supplemented diet (**Figure 6B**). Regarding the posterior intestine, processes related to membrane transport at the second-level classification of KEGG pathways were significantly reduced in fish fed the EOs-supplemented diet (**Figure 6C**), while processes related to the sulfur relay system and naphthalene degradation at the third-level classification of KEGG pathways were significantly enhanced in fish fed the EOs-supplemented diet (**Figure 6D**).

DISCUSSION

The fish intestine is a complex multifunctional organ. In addition to diet digestion and nutrient absorption functions, this organ is critical for other key physiological mechanisms including water and electrolyte balance, endocrine regulation upon digestion and metabolism, and immunity (7), as well as for the establishment of commensal microbiota (2, 14). As one of the main portals of pathogens' entry into the organism, and due to its intricate immune system associated to a lymphoid tissue that allows microbial colonization, the intestine of farmed fish is a target tissue for dietary manipulations (2, 7). We aimed to describe the effect of the dietary combination of garlic, carvacrol, and thymol EOs in the intestinal mucosa of gilthead seabream at a transcriptional level and its impact on gut microbiota modulation.

Effect of Garlic, Carvacrol, and Thymol EOs Additive on Proteolysis, Protein Deubiquitination, and Inflammatory Regulation

The dietary supplementation of EOs affected the regulation of several genes associated with proteolytic pathways, peptidases activity, and protein deubiquitination. In particular, several genes coding for proteasomes (*psma6*, *psmc3*, *psma4*, and

psmb6) and ubiquitin peptidases (*usp4*, *usp10*, *usp48*, *otud3*, and *ufsp2*) were activated by the dietary supplementation of EOs. Protein ubiquitination and subsequent proteolysis and degradation by the proteasome are important mechanisms in the regulation of the cell cycle, cell growth and differentiation, gene transcription, signal transduction, and apoptosis, including tissue regeneration (15). As important factors for the maintenance of intestinal epithelial integrity and immune homeostasis, the ubiquitin-proteasome proteolytic system plays a pivotal role in the activation of the nuclear factor kB (NF-kB) pathway. The NF-kB pathway is involved in the transcriptional regulation of several proinflammatory genes, playing a critical role in regulating the survival, activation, and differentiation of innate and adaptive immune cells (16). As an important feedback regulatory mechanism following NF-kB activation, the NFKB inhibitor alpha (*nfkbia*), also known as *IkBα*, is one of the first genes to be activated (16). In our transcriptional analysis, *nfkbia* was downregulated. In regard to the NF-kB signaling pathway, we also registered the upregulation of several deubiquitination-related genes, such as the referred ubiquitin peptidases, that could be blocking the ubiquitination and degradation of the NF-kB inhibitors. This mechanism might be accompanied by commensal γ-*Protebacteria* strains, the dominant class in our study, that prevent or limit epithelial gut inflammation (17); thus, evidencing the close cooperation between gut and microbiota in the inflammatory regulation in response to dietary shifts. Accordingly, the proinflammatory cytokine interleukin-1 beta (*il-1β*) was downregulated in the fish fed the EOs-supplemented diet, corroborating the reduction of the transcription of proinflammatory genes mediated by the NF-kB pathway. Collectively, these data suggest a direct and selective regulation of the NF-kB and ubiquitin-proteasome pathways established through the interaction between the transcriptome response and their commensal bacteria in the gut of sea bream fed the EOs-supplemented diet.

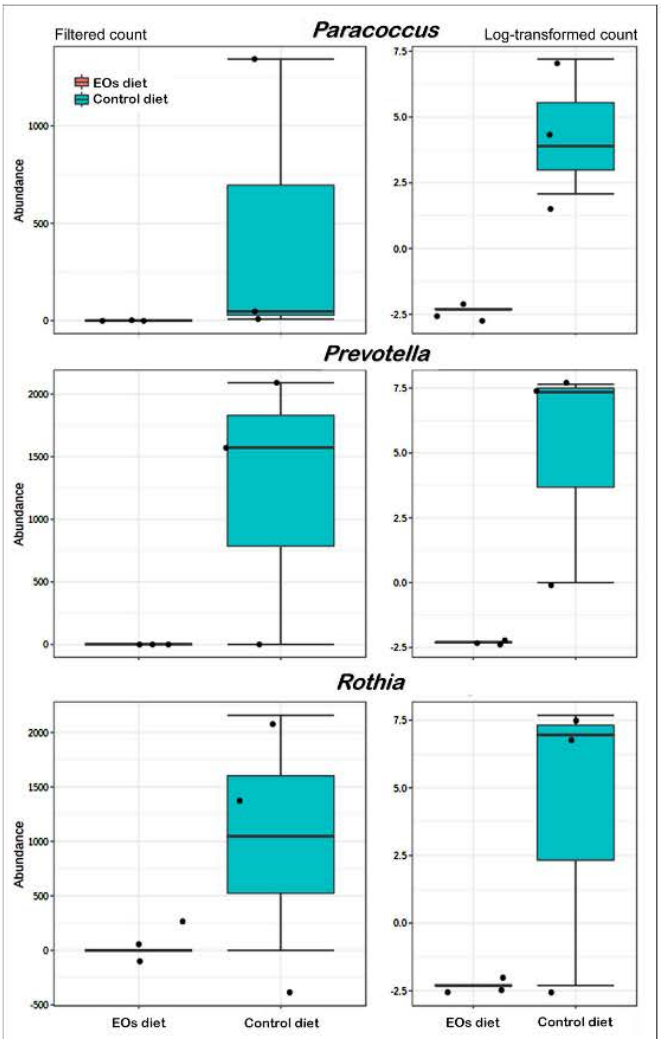

FIGURE 5 | Average abundance of genera showing significant differences ($p < 0.01$) in the posterior intestine of gilthead seabream (*S. aurata*) fed the garlic, carvacrol, and thymol essential oils (EOs)-supplemented diet in comparison with fish receiving the control diet.

Effect of Garlic, Carvacrol, and Thymol EOs Additive on Immune Effector Processes

The dietary administration of EOs showed the modulation of several biological processes related to innate immune effector cells, such as "leukocyte activation," "leukocyte activation involved in immune response," "neutrophil activation," and "neutrophil degranulation." In the case of neutrophils, their main function is the control of microorganisms that cross the epithelium barrier to invade the mucosa. Contrarily to mammals, fish neutrophils are not so abundantly present in the bloodstream, whereas they are stored in hematopoietic reservoirs instead, which could signify a disadvantage for rapid migration and effective resolution of infection and inflammation events (7). Thus, the results of our functional analysis regarding leukocyte activation, and granulocytes, in particular, might suggest an increased intestinal specific immune capacity promoted by the tested functional diet. In fact, the dietary supplementation of garlic or its bioactive compounds (18), carvacrol, and/or thymol (19) have been reported to increase the number of white blood cells and other immune parameters in several cultivated fish species. Similarly to our results, the activation of the degranulation transcriptional response of the neutrophils was previously observed in the gills of gilthead seabream fed the same EOs-supplemented diet and attributed to its increased defense capacity against a monogenean helminth parasite infection (9).

Further analyses on immune-related processes modulated by the tested EOs also showed an increase of the expression in *prex1*, a gene coding for the phosphatidylinositol 3,4,5-trisphosphate-dependent Rac exchanger 1 that regulates adhesion, migration, tissue recruitment, and reactive oxygen species (ROS) formation in the neutrophils (20). In addition, the leukocyte elastase inhibitor gene (*serpinb1*) was also upregulated in the intestine of fish fed the EOs-supplemented diet. This gene encodes a serine protease inhibitor that specifically inhibits neutrophil elastase, cathepsin G, and proteinase-3 present in the neutrophil granules;

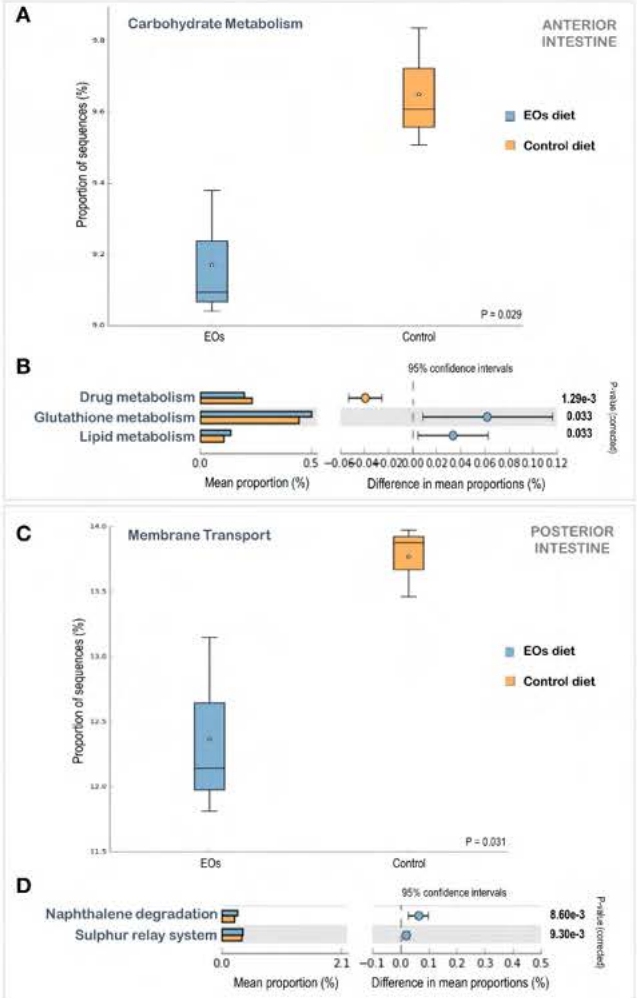

FIGURE 6 | Comparison of the relative abundance of PICRUSt generated functions profile in the anterior **(A,B)** and posterior **(C,D)** intestinal microbiota of gilthead seabream (*S. aurata*) fed the control and the garlic, carvacrol, and thymol essential oils (EOs)-supplemented diet. Box plots show the relative abundances of significant changes in level 2 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways in the anterior **(A)** and posterior **(C)** intestinal microbiota. Box plot central lines indicate the median and star symbols indicate the mean of the data. Significant KEGG pathways at level 3 pathways in the anterior **(B)** and posterior **(D)** intestinal microbiota, by ANOVA with a *post-hoc* Tukey–Kramer multiple-comparison test ($p < 0.05$).

thus, protecting not only tissues from damage at inflammatory sites during stress or infection, but also the neutrophil itself (21). SERPINB1 also limits the activity of inflammatory caspases during inflammation by suppressing their caspase-recruitment domain oligomerization and enzymatic activation, representing an important regulator of tissue inflammation (22). Under current experimental conditions, these results may indicate a well-balanced intestinal immunity, where both immune effector cells activation and an anti-inflammatory response were promoted.

The expression of several genes associated with adaptive immunity was modulated by the EOs-supplemented diet as well. For instance, perforin-1 (*prf1*) was upregulated in the intestine of fish fed the EOs-diet. Perforin is a pore-forming cytolytic protein found in the granules of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells, playing a key role in killing other cells that are recognized as non-self by the immune system (23). In fish, studies have reported the upregulation of *prf1* in response to viral stimulation (24). Furthermore, the upregulation of adenosine deaminase (*ada*) was also promoted by the tested EOs-supplemented diet. In particular, *ada* acts as a positive regulator of T-cell co-activation, participates in the regulation of lymphocyte-epithelial cell adhesion, and enhances dendritic cell immunogenicity (25, 26). Additionally, *cd9* and *cd81* were both upregulated in the fish fed the EOs-supplemented diet. These genes encode tetraspanins, key players in the processes of adhesion, extravasation, and recruitment of leukocytes into inflammation sites, regulating several steps of the immune response (27). CD9 and CD81 were found to be extensively present in Atlantic salmon (*Salmo salar*) IgM^+ B-cells (28). Last but not least, tetraspanins were considered to be required for bacteria adhesion to the epithelial cells (29); which may be in agreement with the presence of both DEGs in the symbiosis-related processes from our transcriptional analysis, as discussed below. Therefore, the regulation of genes involved in both B and T lymphocytes activity may suggest the stimulation of not only the innate, but also the adaptive immune response as well, although further research is needed to confirm this hypothesis.

The proportion of up to downregulated genes related to an immune response was not as marked as the observed for the remaining biological processes activated by the inclusion of EOs in diet, indicating an effective and balanced proinflammatory and anti-inflammatory regulation of the induced immune response, as previously suggested. Nonetheless, it is legitimate to assume that due to its immunostimulatory characteristics, the EOs-based functional feed might have an impact on the composition of the gilthead seabream intestinal microbiota, which in turn may also have played a critical role in mediating the abovementioned immune response. In fact, numerous studies have indicated that diet is an important factor in the modulation of the gut microbiome composition in vertebrates, dictating also the role of that microbiome in fish health status (2). Regarding the EOs tested, garlic (4), carvacrol, and/or thymol (5) were previously reported to modulate fish microbiota composition, exerting beneficial effects (30), and improving significantly its resistance to *Vibrio anguillarum* after intestinal infection and stress challenge (31). The administration of similar functional diets with immunostimulatory and/or antimicrobial properties have been also reported to reduce gut microbial diversity (30); however, in our study, alpha diversity was not significantly altered by the dietary EOs, which may be associated with the heterogeneity of analyzed samples, as observed in CV values for the Chao1 index. Under present experimental conditions, the only phylum that showed significant differences among dietary treatments was the Spirochaetes. This phylum contains important gut pathogenic species, such as *Brachyspira* species, for livestock and humans (32). Nonetheless, the impact of

this phylum modulation upon gilthead seabream intestine homeostasis is not clear yet, and further studies should be performed in order to assess which members of the phylum could be participating in the immune response observed.

Effect of Garlic, Carvacrol, and Thymol EOs Additive on Immune-Related Transport and Secretion Processes

The obtained immune-related biological processes were observed to share 77% of DEGs with the transport and secretion category, which genes were observed to be positively affected, in its majority, by the dietary EOs. The substantial amount of DEGs shared among the two categories clearly indicates a common role in the overall observed transcriptional response. In this sense, similar vesicle-mediated transport processes associated with active biogenesis and neutrophil-mediated immune response were observed in the gills of gilthead seabream fed the same EOs-supplemented diet (9), which seemed to indicate a similar action of this functional additive on different mucosal tissues. Epithelial cells are also directly involved in the initiation of the immune response, such as the one mediated by neutrophils. Accordingly, several genes encoding RAS-related GTPases (*nras*, *rab1a*, *rab5a*, *rab10*, and *arhgap1*), recognized as leading regulators of membrane trafficking directing immunity and inflammation cellular responses (33), were upregulated in the intestine of fish fed the functional feed additive. On the other hand, the hypoxia inducible factor 1 subunit alpha (*hif1a*) was downregulated in the fish fed the EOs-supplemented diet. HIF1a functions as a master transcriptional regulator of the adaptive response to hypoxia and it was observed to be transcriptionally induced by ROS through NF-kB (34), contributing in the intestinal mucosa to inflammatory resolution. The decrease in *hif1a* expression by the EOs dietary administration corroborated once again, that although neutrophil activation and vesicle-mediated transport processes were stimulated in the intestine of fish, inflammation derived from ROS release was probably not occurring.

Furthermore, the serine protease 3 (*prss3*) was another gene positively regulated in the intestine of fish fed the EOs-supplemented diet. This protease is involved in the synthesis of antibacterial substances (35); thus, we hypothesized that *prss3* may be involved in the regulation of intestinal immunity. PRSS3 is also a digestive protease specialized for the degradation of trypsin inhibitors (36). Trypsin inhibitors are anti-nutritional factors found in plant-protein sources that impair diet digestibility and generate digestive and metabolic disorders (37). Although the substitution of fishmeal by plant-derived protein sources was not in the scope of our study, *prss3* upregulation in the EOs-supplemented might indicate that the tested EOs could enhance diet digestibility.

Moreover, the expression of the gene coding for the fatty acid binding protein 6 (*fabp6*) was the most positively affected gene by the dietary EOs. Similarly, FABP6 was significantly increased in the intestine of gilthead seabream fed a combination of carvacrol, thymol, and a prebiotic (38), while this gene was downregulated in response to enteritis induced by a parasitic pathogen (39). Furthermore, FABP6 is involved in the transport of bile acids in ileal enterocytes (40). Besides, the influence of the intestinal microbiota on the activity of FABP6 was suggested in zebrafish, since *fabp6* expression decreased significantly after antibiotic treatment (41). Interestingly, in our study, the abundance of *Bacteroidia* class (Bacteroidetes) decreased significantly in the posterior intestine of fish fed the EOs-supplemented diet. Within the *Bacteroidia* class, *Bacteroides* genus bacterial metabolism of bile acids was observed to modulate gut T-cells homeostasis (42). Particularly, shifts toward the phylum Bacteroidetes including the *Bacteroidia* class coincides with mucosal $CD4^+$ T-cell depletion and enterocyte damage (43). Therefore, the upregulation of *fabp6* and the decrease of *Bacteroidia* class might indicate a modulation of the bile acids secretion by the tested EOs, potentially affecting lipids metabolism. Since bile acids are recognized as signaling molecules between the host microbiota and the innate immunity (44), alterations in its secretion could have a role in our observed immune-related transcriptional response previously discussed. Nevertheless, further studies need to be addressed in order to evaluate the impact of the EOs-based feed additive in the digestive secretions and metabolism of gilthead seabream.

Under the transport and secretion context, gut microbiota mediate the metabolism and transport of dietary xenobiotics through the modulation of metabolites of the host or through microbial secretion (45). Accordingly, our functional analysis of KEGG pathways of the posterior intestine microbiota of individuals fed the EOs-supplemented diet showed a decrease in bacterial sequences related to membrane transport. Considering that membrane transport in prokaryotes is associated with bacterial secretion, this decrease could indicate a lower export of enzymes and bacterial toxins, commonly present in the intestinal tract of different fish species (2), representing a potential beneficial effect of the EOs administration in the gut health.

Effect of Garlic, Carvacrol, and Thymol EOs Additive on the Response to Lipids and Hormones

Under a complex neuroendocrine regulation, the gut microbiota also regulate the metabolism of carbohydrates, lipids, and amino acids, whose composition, in turn, is susceptible to diet, health status, and drugs (46). Formerly, we suggested that the dietary administration of the EOs might affect the secretion of bile acids. If right, this could be inducing a response that could be affecting lipid metabolism and/or steroid hormone signaling. In accordance with this hypothesis, some biological processes related to responses to cyclic compounds, such as lipids and hormones, were positively affected by the inclusion of the EOs-based feed additive in the intestinal mucosa of gilthead seabream. For instance, the ATPase Na^+/K^+ transporting subunit alpha 1 (*atp1a1*) was significantly upregulated in the intestine of fish fed the EOs-supplemented diet. In other studies, diet-induced lipid alterations dramatically affected enterocytes lipid profile in gilthead seabream, reducing significantly Na^+/K^+ ATPase specific activity, suggesting a regulatory role of the lipid microenvironment on the enzyme activity (47). Additionally, tribbles pseudokinase 1 (*trib1*) gene expression was upregulated

in the gilthead seabream fed the EOs-supplemented diet. This gene is known to beneficially affect plasma lipid concentration, playing also major roles in myeloid cells, improving macrophage lipid metabolism, and counteracting inflammation (48). On the other hand, the glutamic-oxaloacetic transaminase 2 (*got2*) gene was downregulated in the fish fed the EOs-supplemented diet. A study in rats suggested that leptin downregulates *got2* in adipocytes (49). Likewise, the adiponectin receptor 1 (*adipor1*) was also downregulated in the fish fed the EOs diet. Adiponectin is an essential hormone predominantly secreted by adipocytes that regulates glucose and lipid metabolism, which along with leptin are considered to be potential proinflammatory adipocytokines (50). Under current experimental conditions, the regulation of genes involved in the cellular response to lipids might suggest the modulation of lipid-related intracellular signaling pathways in the fish fed the EOs-supplemented diet, with a potential role on the immune-inflammatory profile obtained.

Lipids affect the gut microbiota both as substrates for bacterial metabolic processes and by inhibiting bacterial growth by toxic influence (51). In turn, gut microbiota are also pointed as one of the key elements affecting inflammation associated with lipid metabolism dysfunction (2). In fish, the gut microbiota are also recognized to affect considerably the lipid metabolism of the host (41). In agreement to our transcriptional analysis and to the abovementioned findings, the PICRUSt analysis of the microbiota from the anterior intestine of fish fed the EOs-supplemented diet showed a higher abundance of sequences associated with lipid metabolism when compared to the control group. In fact, garlic and its derivatives are widely recognized for their hypolipidemic effect. For instance, one of the primary components of garlic, diallyl disulfide, was suggested to affect both lipid metabolism and gut microbiota in mice through the regulation of the expression of genes associated with lipogenesis and lipid metabolism (52). In other studies, the combined dietary administration of thymol and carvacrol have demonstrated to modulate the intestinal microbiota in piglets, changes that were correlated with an increase in lipid metabolism, among others metabolic effects (53). In this context, our taxonomical analysis at the genus level showed a significant increase in the abundance of *Corynebacterium* (Actinobacteria) in the anterior intestine of fish fed the EOs-supplemented diet. This genus has been reported as a predominant one along the whole digestive tract of gilthead seabream, while its abundance may be modulated by functional diets (54) and dietary lipid levels (55). These results are of special relevance since *Corynebacterium* species are reputed for contributing to manganese acquisition and producing superoxide dismutase and lipases to form organic fatty acids and thioalcohols (56). This genus also showed a higher presence in rainbow trout (*Oncorhynchus mykiss*) intestinal microbiota when the fish were fed high lipid diets (55), evidencing the impact of the tested EOs on the host and microbial lipid metabolism.

Furthermore, a decrease in the abundance of *Rothia* was also detected in the posterior intestine of fish fed the EOs-supplemented diet. *Rothia* abundance was observed to be affected by fish age and sex hormones in gilthead sea bream (14). In effect, the results from our transcriptional analysis revealed a positive regulation of processes related with a response to hormone stimulus. Changes in hormone secretion, such as cortisol, may interfere with the gut immune response (57) and microbiome (58), which could explain the obtained immunity activation and regulation of hormone-sensitive bacteria, such as those belonging to *Rothia* (14). Moreover, stress and stress-related hormones are known to affect carbohydrate, protein, and lipid metabolisms in fish (59), which in turn are also regulated in the host by the gut microbiota. In this sense, a similar feed additive containing garlic and labiatae plant EOs (0.02% inclusion) was demonstrated to reduce significantly plasma cortisol levels in European seabass (*Dicentrarchus labrax*) (60); thus, the potential regulation of stress-related hormones by the tested EOs could explain the response to steroid hormones processes obtained in our functional analysis.

In the present study, the administration of the garlic, carvacrol, and thymol EOs positively affected the expression of growth hormone 2 (*gh2*), although no significant differences in somatic growth were observed at the end of the 65 days of feeding trial. However, GH is not only involved in somatic growth; this hormone also directly stimulates several fish immune factors (61), and participates in the epithelial osmoregulation of euryhaline fish, interacting with cortisol to increase secretory chloride cells and ion transporters involved in salt secretion, such Na^+/K^+ ATPase (62). As a matter of fact, besides immunity and digestion, the gastrointestinal tract of marine teleost fish also plays an important role in osmoregulation. Under this context, the carbonic anhydrase 2 (*ca2*) was the second most positively affected gene by the EOs inclusion in the diet, playing an active role in acid–base regulation through bicarbonate secretion and facilitating epithelial water transport (63). In fact, osmoregulation has been linked to endocrine secretory factors with a significant impact on the fish immune system and microbiota (64).

As previously referred, the *Bacteroidia* class (Bacteroidetes) decreased significantly in the posterior intestine of fish fed the EOs diet. Kan et al. (65) demonstrated that within the *Bacteroidia* class, *Bacteroides* genus abundance increased in goldfish (*Carassius auratus*) when exposed to a toxic environment. Interestingly, our microbiota analysis showed a higher abundance of bacterial 16S rRNA sequences associated with the metabolism of glutathione in the anterior intestine. Glutathione is one of the most important intracellular antioxidant and antitoxin enzymes, whereas its metabolism is regulated by the gut microbiota through the modulation of the amino acid metabolism of the host (66) and tissue oxidative stress (67). Furthermore, glutathione plays important roles in nutrient metabolism and in the regulation of cellular events, such as gene expression, DNA and protein synthesis, cell proliferation and apoptosis, immune response, among others (67). Glutathione S-transferase is one of the key enzymes involved in the second phase of xenobiotics' metabolism and cellular detoxification, catalyzing the conjugation of reduced glutathione to various substances; thus, suggesting a key role in the host immune response modulation (68). Accordingly, in our transcriptional analysis, the glutathione S-transferase theta 2B (*gstt2b*) gene was observed to be upregulated in the intestine of gilthead seabream

following the administration of dietary EOs. The differences observed regarding both transcriptional and microbiota analysis between our experimental diets suggested an improvement of the enterocytes' lipid metabolism and detoxification potential promoted by the additive.

The microbiota analysis also showed a reduction in the proportion of bacterial sequences related to drug metabolism. Accordingly, the EOs-supplemented diet promoted the increase of the cytochrome P450 2J2 (*cyp2j2*) gene transcripts in the intestine of the gilthead seabream. In fish, the cytochrome P450 proteins, and CYP2 family members, in particular, participate in the metabolism of steroidal hormones and other lipids, besides their role in the metabolism of exogenous compounds like drugs and pharmaceuticals (69). Several garlic organosulfur compounds, as well as carvacrol, have been described to selectively modulate the levels of cytochrome P450 genes and proteins (70, 71). Moreover, the mitochondrial peroxiredoxin 3 (*prdx3*) and cathepsin B (*ctsb*) genes were downregulated. Both *prdx3* and *ctsb* are biomarkers of fish stressors (72), whose downregulation might indicate a decrease of the oxidative stress in the fish intestine and a positive impact of the tested additive on fish welfare. Overall, our results indicate that the administrated EOs promotes the enhancement of the antioxidative status in the fish intestine, supporting the gut homeostasis under an immune stimulation scenario.

Effect of Garlic, Carvacrol, and Thymol EOs Additive on the Response to Organic Nitrogen and Aromatic Compounds

In our transcriptional analysis, several genes comprising a response to nitrogenous compounds related processes were also observed to be positively regulated by the presence of garlic, carvacrol, and thymol EOs in the diet. Interestingly, the inclusion of the EOs in the gilthead seabream diet showed a significant decrease in the abundance of the genera *Paracoccus* (Proteobacteria), *Prevotella* (Bacteroidetes) in the posterior intestine, and *Comamonas* (Proteobacteria) in the anterior intestine of fish. All these bacteria are reputed for their capacity for nitrate reduction, as well as being potentially involved in the metabolism of nitrogenous compounds (73). In particular, *Prevotella*, are members of the anaerobic, hydrogen sulfide producing bacterial community (73) that have been previously detected in the intestine of gilthead seabream (54). In humans, an increase in *Pretovella* species at mucosal sites is often associated with chronic inflammation (74). In our study, the PICRUSt analysis showed a lower abundance of predicted carbohydrate degradation pathway in the anterior intestine of fish fed the EOs-supplemented diet, which may be associated with a reduction in *Prevotella* abundance. *Paracoccus* is a genus in the family *Rhodobacteraceae* previously reported in gilthead seabream gut and described as a potential probiotic for this species (75). The relevance of the decrease in the abundance of *Paracoccus* genus needs further investigations in terms of its impact on the condition of the host as no negative effects on gut conditions were observed under present nutritional conditions.

Furthermore, some *Comamonas* strains are also known to have genes for naphthalene degradation (76). The posterior intestine of fish fed the EOs-supplemented diet showed an increase in bacterial sequences related to naphthalene degradation. Naphthalene is an aromatic hydrocarbon present in many EOs with antibacterial, antioxidant, and antiparasitic properties (77). Although suggested to have a positive impact at low concentrations by decreasing DNA damage in some fish species (78), an enhancement in naphthalene and similar compounds degradation is crucial in order to avoid a potential toxicity of the EOs for the host.

The transcriptional analysis showed the positive regulation of the response to alkaloids biological process in the fish fed the EOs-supplemented diet. Alkaloids are versatile heterocyclic nitrogen compounds produced by plants, that along with EOs and phenolic compounds, provide antipathogenic and antioxidant protection (79). This response may not only be associated with the previously referred alteration in the metabolism of nitrogen and carbohydrates induced by the microbiota reshaping, but also with the direct response of the intestinal mucosa to the phenolic monoterpenes carvacrol and thymol (80) and other cyclic compounds derived from garlic (81) with recognized immunomodulatory properties. Moreover, allicin, the main antimicrobial compound in garlic, is also a sulfoxide that bacteria can use in the sulfur-relay system (82). This is in agreement with the observed increase in sequences associated with genes of the sulfur-relay system in the posterior intestine of fish fed the EOs-supplemented diet. Thereafter, considering the complexity of the EOs biochemistry, the transcriptional and bacterial response to those compounds is equally multifaceted. Further studies should be addressed in order to clarify the impact of these potential metabolic alterations in the gilthead seabream gut immune status.

Effect of Garlic, Carvacrol, and Thymol EOs Additive on Symbiosis Processes

The intricate host-microbiota symbiosis in the fish is still substantially unexplored when compared with mammals, and considering its complex challenges to define an "ideal" microbiome for each species since microbiota are strongly modulated by environmental and dietary factors (2). Even though both transcriptional and microbiota modulations by the EOs supplementation were observed, our results fit within the farmed gilthead seabream gut microbiome profile in terms of dominant phyla bacterial composition (14), discarding warnings of a diet-induced dysbiosis. The transcriptomic functional analysis was able to particularly detect such interactions through the expression of several genes related to symbiotic, multi-organism processes, and interspecies interaction between organisms.

For instance, the microbiota taxonomical analysis at the genus level showed an increase in the abundance of *Photobacterium* (Proteobacteria, Vibrionaceae) in the anterior intestine of fish fed the EOs diet. Although some members of this genus, such as *Photobacterium damselae* subsp *piscicida* and *P. damselae* subsp *damselae* have been reported as important pathogens for gilthead seabream (83), they are generally detected in the

intestine of healthy specimens (84, 85). Most species of the *Photobacterium* genus are non-pathogenic and are usually in a symbiotic relationship with marine organisms as enteric commensals. In fact, *Photobacterium* spp. have been even found to be beneficial as a member of the fish intestinal microbiota by its ability to aid with digestion of compounds, such as chitin (86), to produce polyunsaturated fatty acids or even antibacterial secondary metabolites that could inhibit the growth of other pathogenic bacteria (87). This genus has been reported as a member of the intestinal microbiota of marine farmed fish, including gilthead seabream (54, 85), and it has been demonstrated that this genus is one of the most modulated genera in the fish when applying functional diets (88). Regarding the antimicrobial effect of the EOs-supplemented diet, an *in vitro* study demonstrated that the ethanolic extracts of oregano leaves, predominantly composed of carvacrol and thymol, presented a strong bactericidal activity against several pathogens including *Photobacterium damselae,* besides its immunostimulatory effect on gilthead seabream head kidney leukocytes (89). Therefore, our results might suggest a selective antimicrobial effect of the compounds administrated, evidencing the importance of the host-microbiota symbiotic relationship in the modulation of the response to a dietary change.

Additionally, in our transcriptional analysis, the retinoic acid receptor alpha (*rara*) and the retinoic X receptor beta (*rxrb*) genes were both up and downregulated, respectively, in the gut of fish fed the EOs-supplemented diet. The retinoic acid (RA) is the most important transcriptionally active component of the vitamin A, an essential dietary nutrient for fish that plays a significant role in a range of physiological processes including the differentiation and maintenance of epithelial cells and immunity (90). Under this context, another case of symbiotic interaction between organisms is the relation between vitamin A metabolism of the host and its commensal microbiota. Remarkably, *Clostridia* (Firmicutes) abundance was significantly reduced in gilthead seabream fed the EOs diet, which could then be positively affecting the RA availability and the observed regulation of the nuclear receptors (90), potentially participating in the local immunity boost observed in our study. In fact, dietary garlic powder was demonstrated to have an antimicrobial effect on *Clostridium* human bacteria, being suggested to temporarily modulate the gut microbiota (91). In rainbow trout, different levels of garlic extract (1%, 1.5%, and 2%) positively affected the abundance of this genus (4). Curiously, a similar dietary additive composed of garlic and labiatae plants oils was observed to enrich the *Clostridia* class in European seabass fed a low fishmeal and fish oil diet (30). However, carvacrol and thymol, in particular, were numerously observed to exert an antimicrobial effect on *Clostridium* species, proving beneficial for the gut health of several organisms (92); thus, attributing to carvacrol and thymol the main role in the observed reduction of the genus. Given the significance of this symbiosis, the manipulation of RA signaling derived from dietary components acting directly on nuclear receptors and/or on the intestinal microbiota might represent a strategy to promote gut immunostimulation.

Effect of Garlic, Carvacrol, and Thymol EOs Additive on Gene Expression and RNA Processing

Dietary manipulations are widely recognized to directly or indirectly influence the regulation of the fish gut gene expression, in order to reshape its metabolic and physiological responses to different requirements. Indeed, the utmost upregulated biological processes in the intestine of fish fed the functional feed additive tested in our study, in terms of the number of DEGs, were those related to gene expression and processes involved in RNA processing, RNA splicing, mRNA metabolism, and mRNA and ribonucleoprotein export from nucleus. The regulation of gene expression comprises diverse cell mechanisms in order to increase or decrease the production of a specific gene product, either RNA or a protein. For instance, several zinc finger proteins were up (*znf572*, *zeb2*, *znf74*, *zc3h11a*, and *znf214*) and down (*znf133*, *znf551*) regulated in our transcriptional analysis. Besides the stimulation of the transcriptional machinery (93), several genes involved in the spliceosome-mediated splicing (*snrnp200*, *sart1*, *hnrnpu*, and *prpf8*) were also observed to be upregulated by dietary EOs. The spliceosome splicing complex removes intronic non-coding sequences from pre-mRNA to form mature mRNA that can be translated into protein (94).

In another hand, the intestine is *per se* a highly regenerative organ characterized by its continual cell renewal, allowing the epithelium to bear the constant exertion of food digestion, nutrient absorption, and waste elimination (6). Either tissue damage or microbial invasion promotes inflammation and possible DNA damage, so its repair plays a vital role in maintaining genomic integrity during the cell cycle. For instance, DNA damage responses may be induced by proinflammatory cytokines (95), in which transcriptional response appeared not to be promoted by the EOs in our study, as previously discussed. However, genes coding DNA damage checkpoint proteins were up (*fbxo31*, *gltscr2*, *wisp1*, *usp10*, and *cdk5rap3*) and down (*nbn*) regulated by the EOs-supplemented diet, evidencing a regulation of the cell turnover independent from inflammatory stimuli. This hypothesis is reinforced by the upregulation of *cdk5rap3*, the gene encoding CDK5 regulatory subunit associated protein 3, an interactor controlling cell proliferation that among other functions negatively regulates NF-kB mediated gene transcription (96), as initially suggested. Our results also evidence the tight functional connection and coordination between DNA damage responses and immunity, a link that is recognized by its involvement in the protection of the host from infectious microorganisms and surveillance against malignant diseases (97). Therefore, the upregulation of a substantial number of genes that modulates others' expression and that has an implication in transcriptional, translational and DNA repair processes validates the effect of the EOs-supplemented diet on the direct transcriptional regulation of several intestinal cellular processes, including the modulation of the inflammatory and immune response.

CONCLUSIONS

The present complementary analysis of the intestinal transcriptomic profiling and microbiota response to a diet supplemented with garlic, carvacrol, and thymol EOs aimed to take a further step in the evaluation of functional feeds in an attempt to understand how diet-induced shifts can affect the overall gut status of farmed fish from an integrative perspective. This kind of integrative analysis can lead to the "chicken or egg" causality dilemma, and exact mechanisms are still elusive. Nevertheless, the present work suggested that the dietary administration of garlic, carvacrol, and thymol EOs modulated the immune transcriptional response of the mid-anterior intestinal mucosa *per se*, but also its microbiota composition, resulting in complex interactions that resulted in the activation of significant biological processes. Taken together, the combined regulation of the referred pathways could suggest the promotion of an immune reinforcement by the EOs dietary administration *in situ*, most probably induced by host-microbial co-metabolism, which could further attenuate the processes of pathogenesis, putting in evidence the re-adaptation response of the intestinal mucosa to the changes observed in the microbiota composition, and *vice versa*. Moreover, no indications of an inflammation associated with the immunostimulation, which could compromise the intestine integrity, were observed. Since no interference with fish growth was observed, promoted changes in both the intestine mucosa and microbiota were assumed to not significantly affect the gut overall metabolism and nutritional status. Thus, the use of the tested EOs is suggested as a promising alternative to chemotherapeutics to be further evaluated in functional diets under the presence of biotic or abiotic stressors.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

ETHICS STATEMENT

All animal experimental procedures were conducted in compliance with the research protocol approved by the IRTA's Committee of Ethics and Animal Experimentation and in accordance with the Guidelines of the European Union Council (86/609/EU) for the use of laboratory animals.

AUTHOR CONTRIBUTIONS

AE and EG designed and carried out the experiments. Biological samplings were performed by EG, AE, RS, and JF. The transcriptomic data analysis and interpretation were performed by JF, RS, EV-V, and FER-L. MB, IC, and MM carried out the microbiota analyses. YR-C reviewed and validated the methodology used in the study. The study was supervised by EG, FER-L, and LT. JF wrote the original draft. All the authors provided the critical feedback, read, and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.625297/full#supplementary-material

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CHAPTER III

Carvacrol, thymol, and garlic essential oil promote skin innate immunity in gilthead seabream (*Sparus aurata*) through the multifactorial modulation of the secretory pathway and enhancement of mucus protective capacity

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Carvacrol, Thymol, and Garlic Essential Oil Promote Skin Innate Immunity in Gilthead Seabream (*Sparus aurata*) Through the Multifactorial Modulation of the Secretory Pathway and Enhancement of Mucus Protective Capacity

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One of the main targets for the use of phytogenics in aquafeeds is the mucosal tissues as they constitute a physical and biochemical shield against environmental and pathogenic threats, comprising elements from both the innate and acquired immunity. In the present study, the modulation of the skin transcriptional immune response, the bacterial growth capacity in skin mucus, and the overall health condition of gilthead seabream (*Sparus aurata*) juveniles fed a dietary supplementation of garlic essential oil, carvacrol, and thymol were assessed. The enrichment analysis of the skin transcriptional profile of fish fed the phytogenic-supplemented diet revealed the regulation of genes associated to cellular components involved in the secretory pathway, suggesting the stimulation, and recruitment of phagocytic cells. Genes recognized by their involvement in non-specific immune response were also identified in the analysis. The promotion of the secretion of non-specific immune molecules into the skin mucus was proposed to be involved in the *in vitro* decreased growth capacity of pathogenic bacteria in the mucus of fish fed the phytogenic-supplemented diet. Although the mucus antioxidant capacity was not affected by the phytogenics supplementation, the regulation of genes coding for oxidative stress enzymes suggested the reduction of the skin oxidative stress. Additionally, the decreased levels of cortisol in mucus indicated a reduction in the fish allostatic load due to the properties of the tested additive. Altogether, the dietary garlic, carvacrol, and thymol appear to promote the gilthead seabream skin innate immunity and the mucus protective capacity, decreasing its susceptibility to be colonized by pathogenic bacteria.

Keywords: SALT, innate immunity, stress, aquaculture, *Vibrio* infection, teleost fish skin mucus, phytogenic additive, interactome

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INTRODUCTION

Fish infectious diseases are one of the main constraints of the aquaculture sector, representing a serious economic, social, and environmental challenge for the industry (1). Since fish farmers depend on high survival rates and healthy animals, strategies to improve their performance, immune status, and welfare are highly demanded for supporting health management practices that positively impact in the final revenue of the fish farm. On this basis, the development of functional feed additives designed to physiologically support fish to cope with pathogenic and other external challenges intrinsic to aquaculture rearing conditions, represents a promising tool to be implemented in a sustainable and environmentally-responsible aquaculture industry (2).

Functional feeds containing essential oils, one of most commonly used group of phytogenics in aquafeeds, have received increased attention during these last years due to their antimicrobial, immunostimulant, antioxidant, anti-stress, and growth-promoting properties (3–5). Besides essential oils being repeatedly demonstrated to stimulate both humoral and cellular components of the fish innate immunity (6), numerous have been also shown to display a noteworthy antimicrobial activity against a wide range of fish pathogens (7, 8), putting them on the spotlight for the development of sustainable prophylactics. Particularly, phytogenics derived from garlic (*Allium sativum* L., Alliaceae, Liliaceae), oregano (*Oreganum vulgare*, Labiateae), and thyme (*Thymus vulgare*, Labiateae) are among the most studied and administrated, due to their recognized health-promoting properties for aquatic species (6).

One of the main targets for this type of nutritional strategies is the mucosal tissues, due to their importance in the protection against the immediate contact with the environment and potential pathogens. Besides acting as a physical barrier, the mucosal layer also offers a biochemical shield, in which elements from both the innate and acquired immunity are present (9). In particular, the fish skin mucus represents the largest mucosal barrier with its whole epidermis directly exposed to the environment. It is responsible for the first line of defense against external threats, determining pathogen adhesion to the epithelial surfaces (10, 11). Furthermore, skin mucus participates in important physiological processes like osmoregulation, swimming, sensory reception (12), and ecological intra and interspecific interactions (11). Additionally, the fish skin is also characterized by its active mucosal immunity, containing a skin-associated lymphoid tissue (SALT), which is able to respond in case of infection (12, 13). In fact, the immune response described in fish skin against antigen stimulation is similar to other mucosa (14). That immune response involves the secretion of innate immune molecules and the action of specialized cells [(15) and references therein].

Therefore, the improvement of both the epidermal mucus composition and the SALT response to environmental stressors including infective agents by means of dietary tools such as functional feed additives, represents a promising approach for preventing bacterial-induced pathologies in farmed fish. In this context, the aim of the present study was to evaluate the inclusion of a functional feed additive composed by a blend of garlic essential oil, carvacrol, and thymol (the main bioactive compounds of Labiateae plants essential oils) in a standard on-growing diet for gilthead seabream (*Sparus aurata*), assessing its effects on the skin transcriptional response, pathogenic bacterial growth capacity in skin mucus, and fish overall health condition. This species was chosen since it is the most important marine farmed fish species in the Mediterranean with an annual production of 85,385.1 t in 2018 and an economic value of 502,398,000 US$ (16); thus, improving health management strategies based on sustainable dietary approaches for this farmed species is of relevance for this growing industry.

MATERIALS AND METHODS

Diets and Fish Rearing

Juveniles of gilthead seabream were purchased from a Mediterranean fish farm (Piscicultura Marina Mediterránea S.L., Andromeda Group, Valencia, Spain) and on-grown at IRTA–Sant Carles de la Ràpita facilities for research purposes (17). Before the onset of the trial, fish were individually measured in body weight (BW) and standard length (SL) to the nearest 0.1 g and 1 mm, respectively (BW = 40.3 ± 0.1 g; SL = 12.0 ± 0.2 mm). Then, 150 juveniles were randomly distributed among six 450 L tanks (25 fish per tank; initial density = 2 kg m^{-3}; three replicate tanks per experimental group) connected to an IRTAmar® system working under an open-flow regime.

Fish were fed two experimental diets, one devoid of the functional feed additive (control diet) and a second one supplemented with 0.5% of a microencapsulated functional additive containing synthetic garlic essential oil, carvacrol, and thymol (AROTEC-G®, TECNOVIT-FARMFAES, S.L., Spain). Both diets were tested in triplicate tanks and administered for a period of 65 days. Fish were hand-fed two times per day at the daily rate of 3.0% of the stocked biomass, which approached apparent satiation. The control diet was formulated with high levels of marine-derived protein sources (30% fishmeal, 2.5% soluble protein concentrate—CPSP 90® and 2.5 krill meal), containing 46% crude protein, 18% crude fat, and 21.5 MJ/kg gross energy (**Table 1**). Both tested experimental diets were formulated to fulfill the nutritional requirements of juvenile gilthead seabream for summer conditions (18). Diets were manufactured by Sparos Lda. (Olhão, Portugal). In particular, main ingredients were ground (below 250 µm) in a micropulverizer hammer mill (SH1; Hosokawa Micron, B.V., Doetinchem, The Netherlands). Powder ingredients and oils were then mixed according to the target formulation in a paddle mixer (RM90; Mainca, S.L., Granollers, Spain). All diets were manufactured by temperature-controlled extrusion (pellet sizes: 2.0 mm) by means of a low-shear extruder (P55; Italplast, S.r.l., Parma, Italy). Upon extrusion, all feed batches were dried in a convection oven (OP 750-UF; LTE Scientifics, Oldham, UK) for 4 h at 45°C.

The nutritional assay was performed under natural photoperiod (August–September), with daily monitoring of the water temperature (25.1 ± 1.5°C, range: 22.6–28°C), oxygen (6.8 ± 1.7 mg/L; >80% saturation) (OXI330, Crison Instruments, Barcelona, Spain) and pH (7.5 ± 0.01) (pHmeter 507, Crison

TABLE 1 | Formulation of the control diet used during the nutritional assay.

Ingredients	Control diet (%)
Fishmeal 70 LT FF Skagen	20.0
Fishmeal CORPESCA Super Prime	10.0
CPSP 90	2.5
Squid meal	2.5
Soy protein concentrate (Soycomil)	5.0
Wheat Gluten	5.0
Corn gluten	8.0
Korfeed 60	4.5
Soybean meal 48	8.0
Rapeseed meal	4.0
Sunflower meal	3.0
Wheat meal	7.0
Whole peas	2.5
Fish oil–COPPENS	9.0
Soybean oil	1.5
Rapeseed oil	2.5
Vitamin and mineral Premix PV01	2.0
Soy lecithin–Powder	2.0
Antioxidant powder (Paramega)	0.4
Dicalcium phosphate	0.6
Proximate composition, % in dry basis	
Crude protein	46.2
Crude fat	18.4
Gross energy (MJ/kg)	21.5

Instruments). Salinity (35‰) (MASTER-20 T; ATAGO Co. Ltd, Tokyo, Japan), ammonia (0.13 ± 0.1 mg NH_4^+/L), and nitrite (0.18 ± 0.1 mg NO_2^-/L) levels (HACH DR9000 Colorimeter, Hach®, Spain) were weekly measured.

Sampling

At the end of the trial, all fish were anesthetized (buffered 150 mg/L MS-222, Sigma-Aldrich, Madrid, Spain) and measured for individual body weight and standard length ($BW_{control\ diet}$ = 157.8 ± 14.2 g and $SL_{control\ diet}$ = 17.3 ± 0.6 cm; $BW_{supplemented\ diet}$ = 150.8 ± 14.9 g and $SL_{supplemented\ diet}$ = 17.1 ± 0.6 cm) as published in (17). Then, eight fish from each tank (n = 24 per dietary treatment) were randomly selected and skin mucus sample collected following the method described in (19). In brief, skin mucus was collected from the over-lateral line of anesthetized fish in a front to caudal direction using sterile glass slides, and mucus was carefully pushed and collected in a sterile tube (2 mL), avoiding the contamination with blood and/or urine-genital and intestinal excretions. The above-mentioned procedure lasted <2 min in order to avoid the degradation of mucus metabolites. Mucus samples were homogenized using a sterile Teflon pestle to desegregate mucus mesh before centrifugation at 14,000 × g during 15 min at 4°C. The resultant mucus supernatants were collected, avoiding the surface lipid layer, aliquoted, and stored at −80°C for further analysis. For transcriptional analysis purposes, other four fish were randomly selected from each tank (n = 12 fish per dietary treatment) and euthanized with an anesthetic overdose. A ca. 1 cm^2 section of the skin from the mid region of the body over the lateral line of the right side from each fish was dissected, and the muscle tissue attached to it removed. Samples were immersed in RNAlater™ (Invitrogen, Thermo Fisher Scientific, Lithuania), incubated overnight (4°C) and stored at −80°C for further RNA extraction.

Skin Transcriptomic Analysis

RNA Isolation and Quality Control

Total RNA from the skin of twelve randomly selected fish per dietary treatment was extracted using the RNeasy® Mini Kit (Qiagen, Germany). Total RNA was eluted in a final volume of 35 μL nuclease-free water and treated with DNAse (DNA-free™ DNA Removal Kit; Invitrogen, Lithuania). Total RNA concentration and purity were measured using Nanodrop-2000® spectrophotometer (Thermo Scientific, USA) and stored at −80°C until analysis. Prior to hybridization with microarrays, RNA samples were diluted to 133.33 ng/μL concentration, checked for RNA integrity (Agilent 2100 Bioanalyzer; Agilent Technologies, Spain) and selected by the criteria of a RIN value >8.5. Three different pools of samples per dietary treatment were established (n = 4 fish each pool).

Microarray Hybridization and Analysis

Skin transcriptional analysis from both experimental groups was carried out using the Aquagenomics *Sparus aurata* oligonucleotide microarray v2.0 (4 × 44 K) (SAQ) platform. Detailed information and transcriptomic raw data are available at the Gene Expression Omnibus (GEO) public repository at the US National Center for Biotechnology Information (NCBI), accession numbers GPL13442, and GSE162504, respectively. The sampling labeling, hybridization, washes, and scanning was performed as described in (19). Briefly, a one-color RNA labeling was used (Agilent One-Color RNA Spike-In kit; Agilent Technologies, USA). RNA from each sample pool (200 ng) was reverse-transcribed with spike-in. Then, total RNA was used as template for Cyanine-3 (Cy3) labeled cRNA synthesis and amplified with the Quick Amp Labeling kit (Agilent Technologies). cRNA samples were purified using the RNeasy® micro kit (Qiagen). Dye incorporation and cRNA yield were checked (NanoDrop ND-2000® spectrophotometer). Then, Cy3-labeled cRNA (1.5 mg) with specific activity >6.0 pmol Cy3/mg cRNA was fragmented at 60°C for 30 min, and hybridized with the array in presence of hybridization buffer (Gene expression hybridization kit, Agilent Technologies) at 65°C for 17 h. For washes, microarrays were incubated with Gene expression wash buffers, and stabilization and drying solution according to manufacturer instructions (Agilent Technologies). Microarray slides were then scanned (Agilent G2505B Microarray Scanner System), and spot intensities and other quality control features extracted (Agilent Feature Extraction software version 10.4.0.0).

The Search Tool for the Retrieval of Interacting Genes (STRING) public repository version 11.0 (https://string-db.org) was used to generate the skin transcripteractome for the

fish fed the phytogenic-supplemented diet. A Protein-Protein interaction (PPI) Networks Functional Enrichment Analysis for all the differentially expressed genes (DEGs) was conducted with a high-confidence interaction score (0.9) using *Homo sapiens* as model organism (17, 20). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of all the DEGs obtained were also assessed through STRING ($P < 0.05$). In order to confirm match of gene acronyms between both *Homo sapiens* and gilthead seabream species, human orthology identification based on gene/protein name was accessed through the Genecards (www.genecards.org) (21) and Uniprot (www.uniprot.org) databases. Additionally, protein-protein BLAST (BLASTp) were run ($E < 10^{-7}$; query cover > 95%).

Skin Mucus Parameters

Bacterial Growth Assessment in Skin Mucus

Two bacterial fish pathogens were used for the growth curve assay, *Vibrio anguillarum* (CECT number: 522T), and *Pseudomonas anguilliseptica* (CECT number: 899T) from the Spanish Type Culture Collection (CECT, University of Valencia, Valencia, Spain), and the non-pathogenic bacterium for fish, *Escherichia coli* (DSMZ number: 423) from the German Collection of Microorganisms and Cell Cultures (Leibniz Institute DSMZ, Germany). The two pathogenic bacteria were cultured at 30°C for 24 h in marine broth (MB-2216, Becton and Dickinson, USA) and the *E. coli* was cultured at 37°C for 24 h in trypticasein soy broth (TSB, Laboratorios Conda, Spain). For the culture in skin mucus, bacterial suspension with optical density (OD) of 0.2 were centrifuged and the pellet resuspended in sterile PBS, diluted in new growth medium and adjusted to 106 colony-forming units (CFU) mL^{-1}. Then, to study the bacterial growth capacity in the skin mucus, aliquots of 100 µL of the previously cultured bacteria were incubated in 100 µL of skin mucus (3 pools of 6 individual fish per dietary treatment). In parallel, 100 µL of the same cultured bacteria were also incubated in 100 µL of its respective culture media, as a positive control. Triplicates of 100 µL of each fish mucus samples added to 100 µL of culture media were used as negative control and values subtracted from the bacteria–mucus aliquot results. The bacterial growth was measured by absorbance at $\lambda = 400$ nm every 30 min for 14 h at 25°C in flat-bottomed 96-well plates using an Infinity Pro200^{TM} spectrophotometer. Similar temperature values for bacterial growth cultures (25°C) and fish rearing (25.1°C) were chosen in order to standardize mucus analyses with regard to fish rearing conditions used in the nutritional trial.

Skin Mucus Metabolites and Cortisol Analyses

Glucose concentration on fish skin mucus was determined by an enzymatic colorimetric test (LO-POD glucose, SPINREACT®, St. Esteve de Bas, Spain) as described in (19). The OD of the reaction was determined at $\lambda = 505$ nm with a microplate reader and glucose values expressed as µg glucose per mL of skin mucus. Lactate concentration was determined by an enzymatic colorimetric test (LO-POD lactate, SPINREACT®) following the manufacturer's instructions but with slight modifications for fish skin mucus (19). The OD was determined at $\lambda = 505$ nm and lactate values expressed as µg lactate per mL of skin mucus. Protein concentration of previously homogenized mucus samples was determined using the Bradford assay (22) using bovine serum albumin (Sigma Aldrich, Madrid, Spain) as standard. In particular, mucus samples or standard solutions (from 0 to 1.41 mg mL^{-1}) were mixed with 250 µL of Bradford reagent and incubated for 5 min at room temperature. The OD was determined at $\lambda = 596$ nm in a microplate reader. Protein values were expressed as mg protein per mL of skin mucus. Cortisol levels were measured using an ELISA kit (IBL International, Tecan Group, Switzerland) following the manufacturer's instructions for saliva determinations. Values of OD were determined at $\lambda = 450$ nm with a microplate reader. Cortisol values were expressed as ng cortisol per mL of skin mucus. All standards and samples were analyzed in triplicate (methodological replicates) and spectrophotometric measurements were conducted with an Infinity Pro200^{TM} spectrophotometer (Tecan, Männedorf, Switzerland).

Mucus ratios referred to protein (glucose/protein, lactate/protein and cortisol/protein) were calculated in order to avoid the putative dilution or concentration derived from mucus sampling. As an indicator of the metabolic aerobic response, the glucose/lactate ratio was also calculated (19).

Ferric Antioxidant Power (FRAP) was measured by means of an enzymatic colorimetric test (Ferric antioxidant status detection kit, Invitrogen, Thermo Fisher Scientific, Spain), following the manufacturer's instructions for plasma, with minor modifications. Briefly, 20 µL of mucus sample or standard solutions (from 0 to 1,000 µM μL^{-1} of $FeCl_2$) were mixed with 75 µL of FRAP color solution and incubated at room temperature for 30 min, in triplicate. The OD was measured at $\lambda = 560$ nm. Antioxidant values were expressed as nmol FRAP per mL of mucus, and nmol FRAP per mg of mucus protein. All measurements were performed with a microplate spectrophotometer reader (Infinity Pro200^{TM} spectrophotometer).

Statistical Analysis

Differences between growth performance parameters were analyzed through an unpaired *t*-test ($P < 0.05$) with GraphPad PRISM 7.00 assuming data homoscedasticity. Differences between skin mucus metabolites and cortisol, and differences in bacterial growth inhibition between the two dietary treatments were assessed with SPSS Statistics for Windows, Version 22.0 (IBM Corp, Armonk, NY, USA) through an unpaired *t*-test ($P < 0.05$). Microarrays extracted raw data were imported and analyzed with GeneSpring version 14.5 GX software (Agilent Technologies). The 75% percentile normalization was used to standardize arrays for comparisons and data were filtered by expression. An unpaired *t*-test was conducted without correction to identify those DEGs between both dietary treatments. A $P < 0.05$ was considered statistically significant. The representation for the principal component analysis (PCA) and the hierarchical heatmap were generated using GeneSpring version 14.5 GX software.

RESULTS

Skin Transcriptomic Profile

Under present experimental conditions, in order to determine the modulatory effect of the dietary supplementation of a blend of garlic essential oil, carvacrol, and thymol upon the skin transcriptome, a microarray-based transcriptomic analysis was conducted in gilthead seabream. In total, 534 differentially-expressed genes (DEGs) were found in the skin from both experimental groups ($P < 0.05$; **Supplementary Table 1**). Among these, 393 genes were up-regulated with 390 belonging to the 1.0–1.5-fold change (FC) interval. The other 3 DEGs were grouped in the $1.5 \leq FC \leq 2.0$ interval. On the other hand, 141 genes were down-regulated ($P < 0.05$) and grouped in the range of $-1.5 \geq FC \geq -1.0$. Although genes were observed to be mostly up-regulated in the group fed with the blend of phytogenics (73.6% of DEGs), gene modulation was moderated in terms of fold-change intensity (**Figure 1A**). Common segregation among the pool samples within the same dietary treatment was observed in the hierarchical clustering for the skin transcriptomic response based in similitude patterns of the DEGs response ($P < 0.05$) (**Figure 1B**). The observed differential profile among dietary treatments is supported by the PCA analysis for the analyzed samples (**Figure 1C**).

When considering the complete list of annotated DEGs, a functional network (transcripteractome) containing 203 nodes was generated (**Figures 2, 3**), which resulted in 341 interactions (edges). The remaining 331 DEGs, annotated as unknown genes, were excluded from the analysis. The enrichment analysis identified in the transcripteractome two main representative processes that were considered to encompass the several Gene Ontology (GO) annotations obtained (**Supplementary Table 2**), denoted as (a) Transcription Regulation, and (b) Secretory Pathway.

The "RNA processing" biological process (GO:0006396; 12 up-regulated genes; 14 down-regulated genes) was the exclusive differentially regulated GO term for the skin of fish fed the diet supplemented with the additive (**Figure 2**). Molecular functions "protein binding" (GO:0005515) and "protein-containing complex binding" (GO:0044877) were also obtained.

In order to elucidate the location relative to the cellular structures in which the DEGs perform their function, several cellular components were identified in the functional network, representing the association between them (**Figure 3**). The analysis included the, "ribonucleoprotein complex" (GO:1990904; 10 up-regulated genes; 9 down-regulated genes), "vesicle" (GO:0031982; 20 up-regulated genes; 20 down-regulated genes), "transport vesicle membrane" (GO:0030658; 2 up-regulated genes; 5 down-regulated genes), "COPII-coated ER to Golgi transport vesicle" (GO:0030134; 2 up-regulated genes; 3 down-regulated genes), "Golgi-associated vesicle" (GO:000579810; 3 up-regulated genes; 4 down-regulated genes), "endosome" (GO:0005768; 10 up-regulated genes; 9 down-regulated genes), "vacuole" (GO:0005773; 9 up-regulated genes; 12 down-regulated genes), "lysosome" (GO:0005764; 7 up-regulated genes; 7 down-regulated genes), and "proton-transporting two-sector ATPase complex" (GO:0016469; 1 up-regulated genes; 3 down-regulated genes).

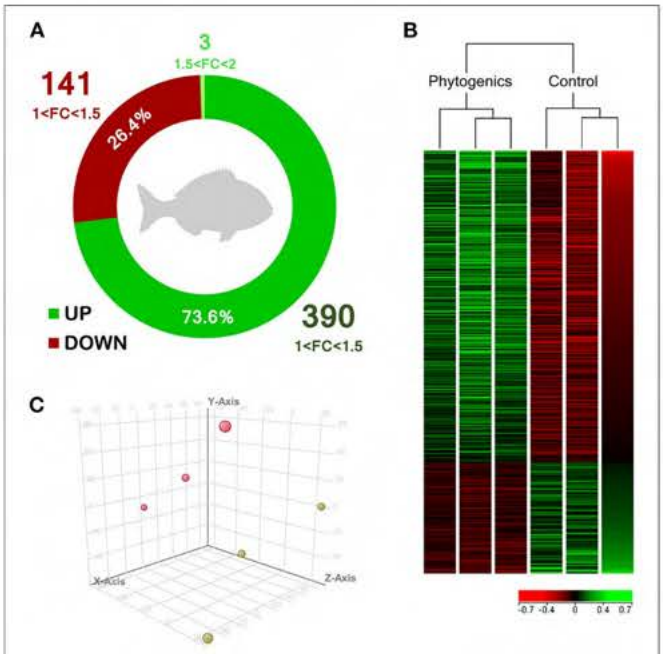


FIGURE 1 | Skin transcriptomic profile of gilthead seabream (*Sparus aurata*) fed a diet supplemented with a blend of garlic essential oil, carvacrol and thymol. **(A)** Differential expression analysis of the gilthead seabream skin transcriptomic response fed a diet supplemented with a blend of garlic, carvacrol and thymol. **(B)** Hierarchical clustering for the control and phytogenic-supplemented diets, based in similitude patterns of the differentially expressed genes (DEGs) detected from three sample pools per dietary group. Data of the six microarrays are depicted, one for each represented pool. Both increased and decreased gene expression pattern is shown in green and red, respectively. All transcripts represented are statistically significant ($P < 0.05$). **(C)** Principal component analysis (PCA) of the DEGs of gilthead seabream skin response to the control diet (yellow) and phytogenic-supplemented diet (pink). Please refers to **Supplementary Table 1** for details.

In order to identify the pathways significantly impacted by the total DEGs obtained, the functional analysis of KEGG pathways revealed also significant differences in the regulation of genes associated with "protein processing in endoplasmic reticulum" (hsa04141; 3 up-regulated genes; 5 down-regulated genes), "phagosome" pathway (hsa04145; 3 up-regulated genes; 5 down-regulated genes), and "*Vibrio cholerae* infection" pathway (hsa05110, belonging to the "infectious disease: bacterial" group; 1 up-regulated gene; 4 down-regulated genes) in the skin of the group fed with the blend of tested phytogenics (**Figure 4** and **Supplementary Table 3**).

Additionally, from the total DEGs obtained from the skin transcriptomic profile of fish fed the phytogenic-supplemented diet, a set of genes were selected by their involvement in the "immune system process" (GO:0002376; 12 up-regulated genes; 5 down-regulated genes). Among the processes related to immunity, "antigen processing and presentation of exogenous peptide antigen via MHC class I" (GO:0002479; 1 up-regulated genes; 2 down-regulated genes), "leukocyte activation"

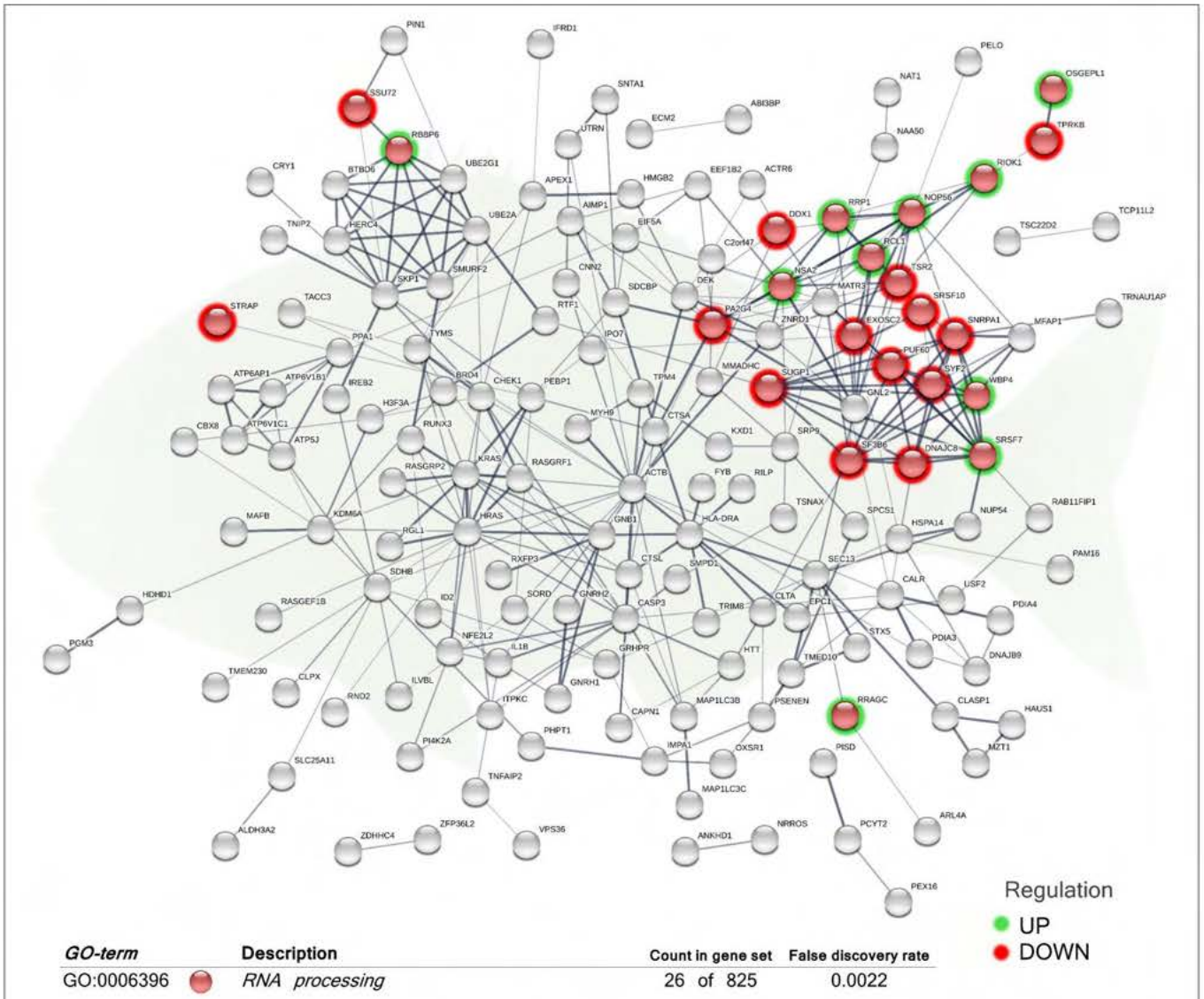


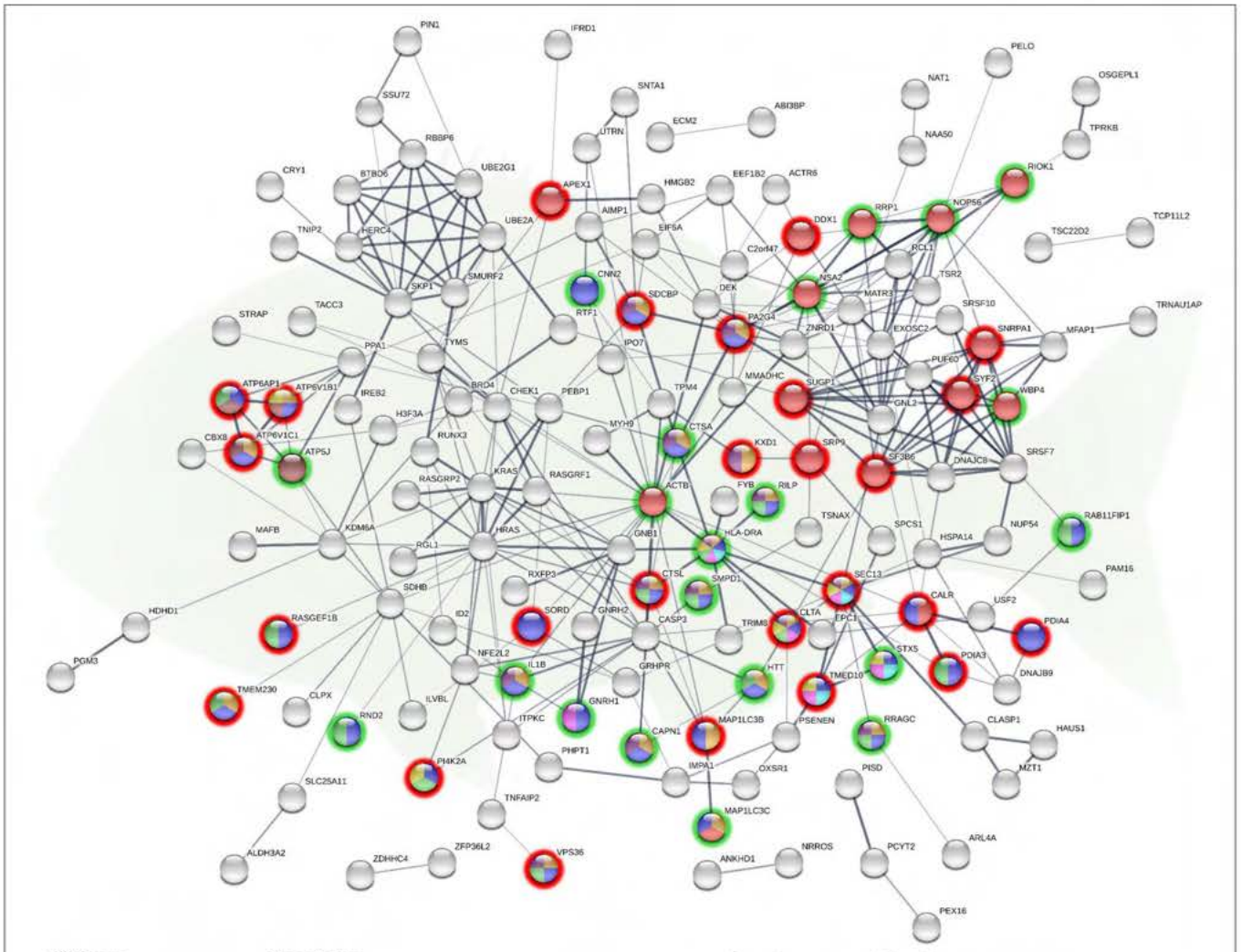
FIGURE 2 | Functional biological network of the differentially expressed genes (DEGs) in the skin of juvenile gilthead seabream (*Sparus aurata*) fed a diet supplemented with a blend of garlic essential oil, carvacrol, and thymol. Green-shaded nodes represent up-regulated genes and red-shaded nodes represent down-regulated genes. Graphic keys and network stats: number of nodes = 203; number of edges = 341; average node degree = 3.36; average local clustering coefficient = 0.4; expected number of edges = 286; PPI enrichment *p*-value = 0.000807. Gene Ontology (GO) definition, count of DEGs within the biological process and respective false discovery rate are described in the graphical figure legend (bottom). For details, please refers to **Supplementary Table 2**.

(GO:0045321; 6 up-regulated genes; 1 down-regulated genes), "regulation of NIK/NF-kappaB signaling" (GO:1901222; 2 up-regulated genes; 1 down-regulated genes), "positive regulation of T cell cytokine production" (GO:0002726; 2 up-regulated genes; 0 down-regulated genes), and "regulation of T cell proliferation" (GO:0042129; 2 up-regulated genes; 1 down-regulated genes) biological processes were highlighted (**Figure 5** and **Supplementary Table 4**).

Bacterial Growth Capacity on Skin Mucus

The transcriptome response arose the modulation of genes associated to immune processes involved in the response to infectious bacterial diseases. Thus, we evaluated whether such response implies a functional protective mechanism against pathogenic bacterial growth on skin mucus. Considering that our data registered a specific response to *Vibrio*, we included in our analysis the fish pathogen *V. anguillarum*. In addition, we also included as control *P. anguilliseptica* (another pathogenic marine fish bacteria) and *E. coli* as non-pathogenic fish bacterium.

When cultured with the skin mucus from fish fed the phytogenic-supplemented diet, a reduction on the growth of the pathogenic bacteria *V. anguillarum* was observed (*t*-test; $P < 0.05$; **Figures 6A,B**). Growth decrease was recorded between 4 and 14 h of bacterial culture; the most accentuated decrease in growth values compared with control diet (over 30%) were found between 8 and 12 h (**Figures 6A,B**). Regarding



GO-term	Description	Count in gene set	False discovery rate
GO:1990904	Ribonucleoprotein complex	19 of 770	0.012
GO:0005773	Vacuole	21 of 682	0.001
GO:0031982	Vesicle	40 of 2318	0.017
GO:0030134	COPII-coated ER to Golgi transport vesicle	5 of 75	0.024
GO:0005798	Golgi-associated vesicle	7 of 155	0.024
GO:0030658	Transport vesicle membrane	7 of 181	0.044
GO:0005768	Endosome	19 of 876	0.033
GO:0005764	Lysosome	14 of 582	0.044
GO:0016469	Proton-transporting two-sector ATPase complex	4 of 49	0.032

Regulation
UP
DOWN

FIGURE 3 | Functional network representing cellular components related to secretory pathways of the differentially expressed genes (DEGs) in the skin of juvenile gilthead seabream (*Sparus aurata*) fed a diet supplemented with a blend of garlic essential oil, carvacrol and thymol. Node colors indicate the cellular component for each DEG represented. Green-shaded nodes represent up-regulated genes and red-shaded nodes represent down-regulated genes. Graphic keys and network stats: number of nodes = 203; number of edges = 341; average node degree = 3.36; average local clustering coefficient = 0.4; expected number of edges = 286; PPI enrichment *p*-value = 0.000807. Gene Ontology (GO) definition, count of DEGs within the biological process and respective false discovery rate are described in the graphical figure legend (bottom). For details, please refers to **Supplementary Table 2**.

P. anguilliseptica, a decline in bacterial growth was observed in both gilthead seabream skin mucus samples from fish fed the control and phytogenic-supplemented diets (**Figures 6C,D**). However, *P. anguilliseptica* growth decline was observed to be more accentuated in the mucus from fish fed the phytogenic-supplemented diet than in that of the control group at 12–14 h of culture (*t*-test; $P < 0.05$), with a maximum decrease in growth of 50.2 ± 1.6% at 14 h (**Figure 6D**).

Gilthead seabream mucus from both nutritional groups showed a decrease of *E. coli* growth during all the culture period (**Figures 6E,F**), though the most emphasized bacterial growth decrease was observed in the initial interval of 4–8 h

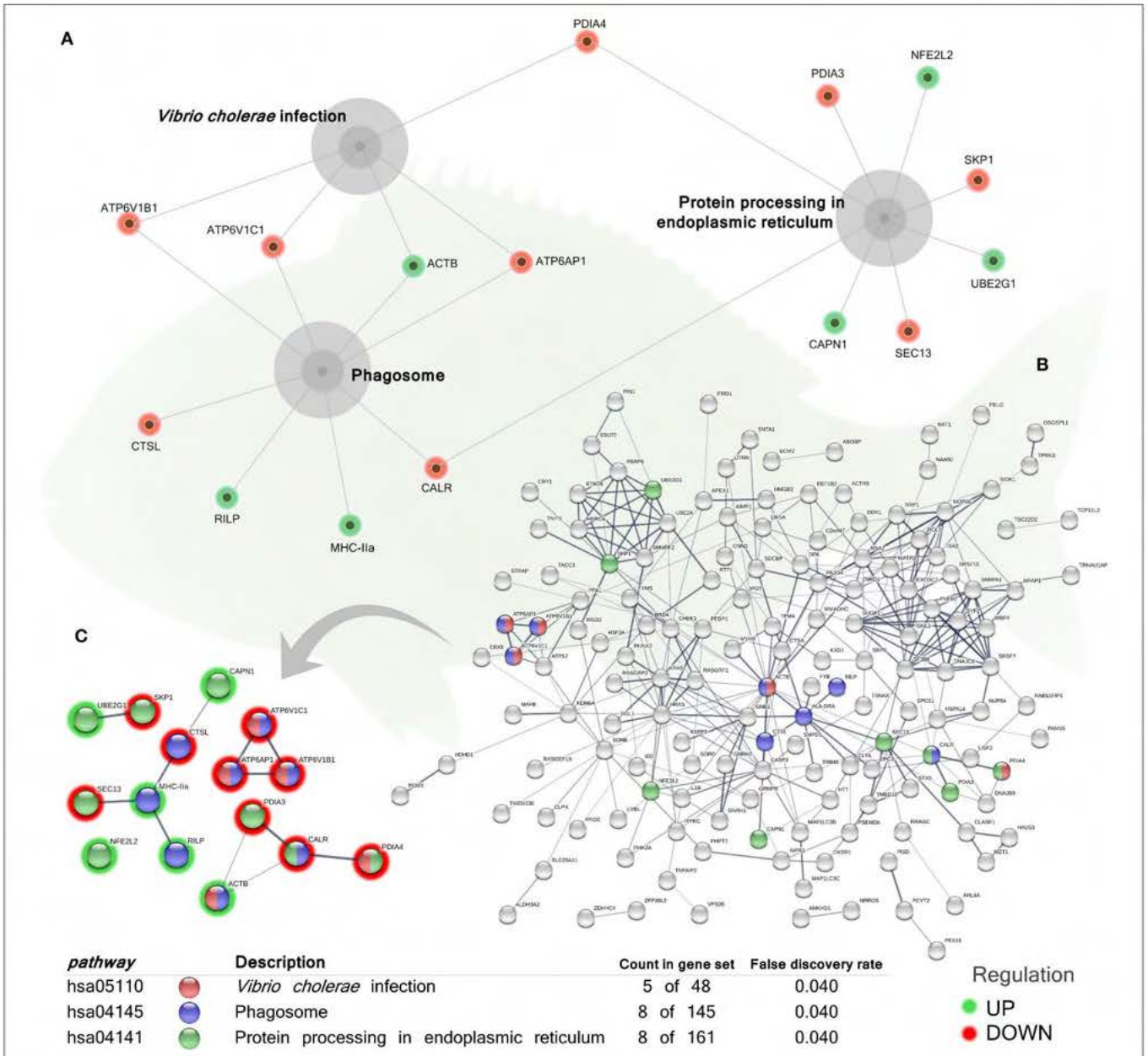
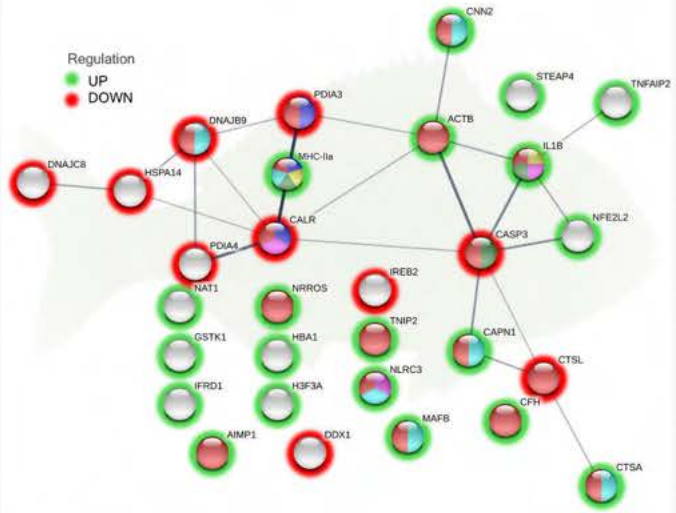


FIGURE 4 | Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways networks of the differentially expressed genes (DEGs) in the skin of juvenile gilthead seabream (*Sparus aurata*) fed a diet supplemented with a blend of garlic essential oil, carvacrol and thymol. **(A)** Gray cores represent the KEGG pathways significantly impacted by the DEGs obtained from the skin of fish fed the phytogenic-supplemented diet. Green-shaded nodes represent up-regulated genes and red-shaded nodes represent down-regulated genes. **(B)** Nodes colors indicate the KEGG pathways for each DEG represented within the overall transcripteractome. Pathways description, count of DEGs within each pathway and respective false discovery rate are described in the graphical figure legend. **(C)** Interactions exclusively among the DEGs within each KEGG pathway. Nodes colors indicate the KEGG pathways for each DEG represented. Pathways description, count of DEGs within each pathway and respective false discovery rate are described in the graphical figure legend. Green-shaded nodes represent up-regulated genes and red-shaded nodes represent down-regulated genes. For details, please refers to **Supplementary Table 3**.

with decreased growth value of 60.3 ± 2.5%, reducing gradually (**Figure 6F**).

Mucus Stress Biomarkers

The skin mucus stress-related biomarkers and their ratios, as well as the ferric antioxidant power are summarized in **Table 2**. The content of soluble protein was not significantly affected by the functional feed additive (t-test, $P > 0.05$). However, glucose, lactate and cortisol levels were observed to be significantly lower in the mucus of gilthead seabream fed the phytogenic-supplemented diet (t-test, $P < 0.05$). No differences among dietary groups were observed in terms of the ferric skin mucus



GO-term	Description	Count in gene set	False discovery rate
GO:0002376	Immune system process	17 of 2370	1.07e-05
GO:0002479	Antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-dependent	3 of 25	0.0037
GO:0045321	Leukocyte activation	7 of 894	0.024
GO:0002726	Positive regulation of T cell cytokine production	2 of 19	0.0265
GO:1901222	Regulation of NIK/NF-kappaB signaling	3 of 97	0.0265
GO:0042129	Regulation of T cell proliferation	3 of 151	0.0496

Gene description	Gene acronym	FC	P
Major Histocompatibility Complex, Class II, Alpha	*mhc-IIa*	1.493	0.029
STEAP4 Metalloreductase	*steap4*	1.39	0.0496
MAF BZIP Transcription Factor B	*mafb*	1.288	0.0042
Hemoglobin Subunit Alpha 1	*hba1*	1.276	0.0421
TNFAIP3 Interacting Protein 2	*tnip2*	1.265	0.0295
Interferon Related Developmental Regulator 1	*ifrd1*	1.226	0.0044
Small Inducible Cytokine Subfamily E Member 1	*aimp1*	1.223	0.001
NLR Family CARD Domain Containing 3	*nlrc3*	1.217	0.0258
TNF Alpha Induced Protein 2	*tnfaip2*	1.195	0.0206
Interleukin 1 Beta	*il1b*	1.192	0.0186
H3 Histone Family Member 3A	*h3f3a*	1.151	0.0182
Calponin 2	*cnn2*	1.138	0.0497
Negative Regulator of Reactive Oxygen Species	*nrros*	1.136	0.0425
Complement Factor H	*cfh*	1.119	0.0294
Nuclear Factor, Erythroid 2 Like 2	*nfe2l2*	1.116	0.0204
Calpain 1	*capn1*	1.105	0.0393
Glutathione S-Transferase Kappa 1	*gstk1*	1.097	0.0357
N-Acetyltransferase 1	*nat1*	1.091	0.0358
Cathepsin A	*ctsa*	1.061	0.049
Actin Beta	*actb*	1.048	0.0357
Protein Disulfide Isomerase Family A Member 3	*pdia3*	-1.09	0.0125
Protein Disulfide Isomerase Family A Member 4	*pdia4*	-1.1	0.0289
Iron-Responsive Element-Binding Protein 2	*ireb2*	-1.102	0.0153
Heat Shock Protein Family A (Hsp70) Member 14	*hspa14*	-1.103	0.0145
DEAD-Box Helicase 1	*ddx1*	-1.11	0.0302
DnaJ Heat Shock Protein Family (Hsp40) Member C8	*dnajc8*	-1.114	0.0024
Cathepsin L	*ctsl*	-1.158	0.0074
Caspase 3	*casp3*	-1.187	0.0137
Calreticulin	*calr*	-1.204	0.0138
DnaJ Heat Shock Protein Family (Hsp40) Member B9	*dnajb9*	-1.205	0.0105

FIGURE 5 | Functional biological network of differentially expressed genes (DEGs) recognized by their involvement in the immune response in the skin of juvenile gilthead seabream (*Sparus aurata*) fed a diet supplemented with a blend of garlic essential oil, carvacrol, and thymol. Green-shaded nodes represent up-regulated genes and red-shaded nodes represent down-regulated genes. Graphic keys and network stats: number of nodes = 30; number of edges = 24; average node degree = 1.6; average local clustering coefficient = 0.4; expected number of edges = 9; PPI enrichment *p*-value = 1.25e-05. Gene Ontology (GO) definition, count of DEGs within the biological process and respective false discovery rate are described in the graphical figure legend (bottom left). For details, please refers to **Supplementary Table 4**. Gene description, respective acronym, fold-change intensity (FC), modulation (color scale), and P-value are described (right).

antioxidant power (*t*-test, $P > 0.05$). When data on skin mucus biomarkers were normalized with the protein content of the sample, only cortisol levels (cortisol/protein) were observed to be significantly reduced in skin mucus from fish fed the phytogenic-supplemented diet when compared to the control diet (*t*-test, $P < 0.05$). Metabolic aerobic response, measured in mucus as glucose/lactate ratio, was neither significantly affected by the dietary treatment.

DISCUSSION

The fish skin mucosal surface is in direct contact with the aquatic environment and represents the first line of defense against external threats, determining pathogen adhesion to the epithelial surface (10, 12). As a mucosal tissue, the skin is characterized by its ability to produce mucus, which apart from being an intrinsic physical barrier, it contains glycosaminoglycans, lectins, antibacterial enzymes, immunoglobulins, and several structural, metabolic, stress-related, and signal transduction proteins [(23–25); among others]. In addition to mucus continuous secretion and replacement (26), its components provide an impermeable capacity against most bacteria and other pathogens, immobilizing them and inhibiting their proliferation before they can contact epithelial surfaces (10). Importantly, an intimate crosstalk between skin tissue and its exuded mucus in response to stimulus has been recently proposed (27), reinforcing the coordinated response capacity that takes place in this mucosal-associated lymphoid tissue.

In the present study, we evaluated the protective benefits of a blend of dietary garlic essential oil, carvacrol, and thymol on gilthead seabream in terms of skin transcriptome and skin mucus secretions. The potential increased skin mucus protective capacity observed in our gilthead seabream fed with phytogenic-supplemented diet could be attributed to the exudation of a variety of biologically active substances and several molecules of the innate and acquired immune system. In fact, many of them have an already reported biostatic and biocidal activities

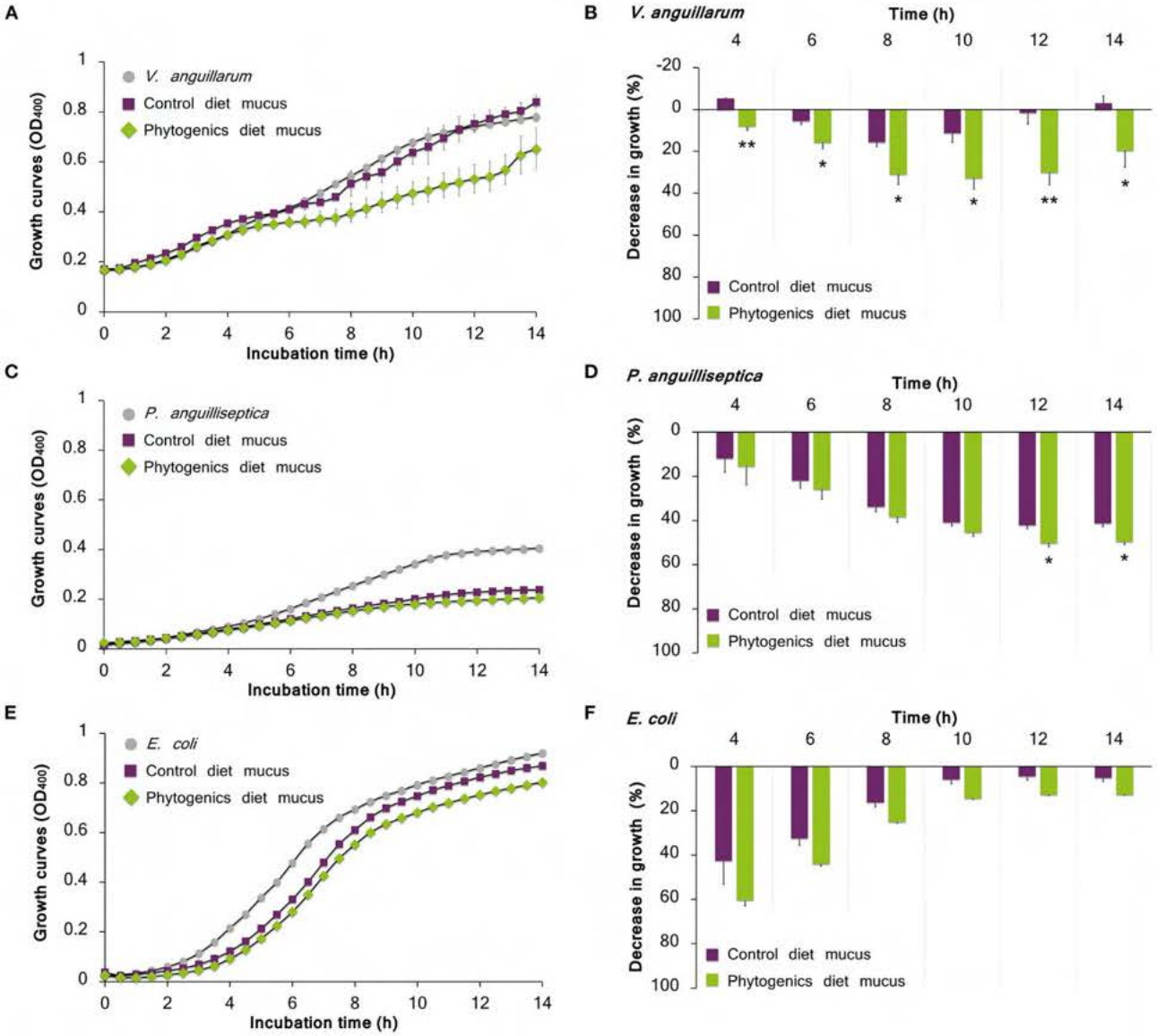


FIGURE 6 | Bacterial growth on skin mucus of juvenile gilthead seabream (*Sparus aurata*) fed a diet supplemented with a blend of garlic essential oil, carvacrol and thymol, and a control diet devoid of the feed additive. Mucus samples were obtained from 3 pools of 6 individual fish. Data correspond to the mean ± SEM of triplicate bacterial growth curves of *V. anguillarum* **(A)**, *P. anguilliseptica* **(C)** and *E. coli* **(E)**. Gray circles correspond to bacteria growth in medium devoid of mucus; purple squares correspond to bacteria growth in the mucus of fish fed the control diet; and green rhombus correspond to bacteria growth in the mucus of fish fed the phytogenic-supplemented diet. Details on the statistical differences in bacterial growth between the experimental dietary groups is provided by the percentage of decrease in growth of *V. anguillarum* **(B)**, *P. anguilliseptica* **(D)**, and *E. coli* **(F)**. Asterisks indicate significant differences in bacterial growth between dietary groups (*t*-test, $P < 0.05$).

(28). Under this context, our transcriptomic analysis for the skin revealed that several genes coding proteins potentially involved in an enhanced skin mucosal protective capacity. For instance, the H3 Histone Family Member 3A gene (*h3f3a*) was up-regulated in the skin of fish fed the phytogenic-supplemented diet. Histones, full-length proteins and/or fragments, are recognized antimicrobial molecules (29), which are found in the mucus of several fish species (30, 31), including gilthead seabream (32, 33). Calpain 1 (*capn1*), a non-lysosomal cysteine protease, was observed to be up-regulated by the functional additive as well. Several calpain proteins were mapped in gilthead seabream's epidermal mucus (34) while its presence in the skin mucus of cod (*Gadus morhua*) was suggested to be a key protective element against *V. anguillarum* infection (35).

Iron is an essential micronutrient required for most bacteria to grow; in the host, this metal is associated to iron-binding proteins, such as transferrin found in fish skin and mucus (23, 30, 32), limiting its availability to invading pathogenic bacteria

TABLE 2 | Skin mucus stress biomarkers of gilthead seabream fed an experimental diet supplemented with a blend of garlic essential oil, carvacrol and thymol, and the control diet devoid of the feed additive ($n = 24$ per dietary treatment).

	Diet	
	Control	**Phytogenics**
Skin mucus stress biomarkers		
Protein (mg/mL)	16.73 ± 2.22	12.86 ± 1.77
Glucose (μg/mL)	13.43 ± 3.95	6.88 ± 1.63*
Lactate (μg/mL)	9.80 ± 2.62	3.62 ± 1.09*
Cortisol (ng/mL)	3.47 ± 0.85	0.35 ± 0.05*
FRAP (μmol/mL)	1,923 ± 244	1,790 ± 315
Skin mucus stress biomarkers ratios		
Glucose/Protein (μg/mg)	0.74 ± 0.16	0.60 ± 0.17
Lactate/Protein (μg/mg)	0.46 ± 0.05	0.31 ± 0.12
Glucose/Lactate (mg/mg)	0.69 ± 0.14	0.77 ± 0.15
Cortisol/Protein (ng/g)	208 ± 48.80	31.85 ± 7.03**
FRAP/Protein (μmol/mg)	114 ± 16.9	139 ± 23.1

Asterisks indicate significant differences between experimental diets ($P < 0.05$, **$P < 0.01$; t-test).*

(36). For instance, *V. anguillarum* iron-uptake system is crucial for sequestering iron from these proteins and accomplishing skin colonization and penetration (37). Interestingly, some genes related with iron metabolism were observed to be down-regulated in the skin of fish fed the functional diet. In our transcriptional analysis, the Iron-Responsive Element-Binding Protein 2 (*ireb2*) gene was down-regulated in fish fed the phytogenic-supplemented diet. When cell's iron levels are low or depleted, this RNA-binding protein binds to iron-responsive elements, found for instance in transferrin mRNAs, regulating the translation and stability of those mRNAs and consequently regulating iron availability (38). In zebrafish, the increase of *ireb2* expression in spleen was proposed to be linked to an augmented iron uptake from *V. anguillarum* (39). Hence, the down-regulation of *ireb2* observed in the skin of fish fed the phytogenic-diet could be suggesting a decrease in the cellular iron uptake as a consequence of the reduction of pathogenic bacteria load in the mucosal tissue. This hypothesis is supported by the decreased growth capacity for pathogenic bacteria in skin mucus observed in the fish fed the tested functional diet.

On the other hand, iron plays an important role in hemoglobin production. The Hemoglobin Subunit Alpha (*hba1*) was up-regulated in the skin of fish fed the phytogenic-supplemented diet. Hemoglobin functionality is not restricted to oxygen transport, since it also binds to pathogen-associated molecular patterns (PAMPs), triggering an immune Toll-like receptor (TLR)-mediated signal transduction (40). In addition, the STEAP4 Metalloreductase (*steap4*) transcripts increased by the phytogenic-diet. This metalloreductase is involved in iron and copper homeostasis, playing also a role in the protection against inflammatory-mediated cellular damage (41). In Atlantic salmon (*Salmo salar*) skin, *steap4* was up-regulated by dietary phytogenics, which was associated to iron sequestration, inflammation and an increased protective capacity against lice infection (42). The *hba1* and *steap4* up-regulation in the skin observed in our study may be implicated in a reduction in iron availability in the skin surface and mucus of fish fed the phytogenics, hampering pathogenic bacteria growth as observed in the potential mucus antibacterial capacity observed in our study.

Fish SALT is characterized by both humoral and cellular components which intimately communicate to mount an immune response in which both innate and adaptive defense mechanisms are involved (15). For instance, the Interferon Related Developmental Regulator 1 (*ifrd1*) was up-regulated by the functional diet. *Ifrd1* gene encodes a protein related to interferon family. In fish is widely recognized by its involvement in the innate immune antiviral response (43), while it is also highly expressed in differentiating neutrophils, playing an important role in neutrophils effector function (44). An increase of *ifrd1* transcripts was observed in the skin of zebrafish (*Danio rerio*) infected by *Aeromonas hydrophila* (45), whereas its up-regulation was also related to its role in the immediate response of the fish immune system to stress (46). Moreover, transcripts of the Complement Factor H (*cfh*) increased in the skin of gilthead seabream fed the phytogenic-supplemented diet. CFH, a major regulator of the complement system, is essential for directing the complement system toward pathogen-related infections, since its transcription is induced by lipopolysaccharide (LPS) (47), whereas it increases the contact between neutrophils and pathogens, increasing cell's phagocytosis capacity and antimicrobial activity (48). CFH is also reputed for protecting host cells and tissues from the self-innate immunity (49). This occurs due to the interaction of the factor H with the C3 convertase and the C3b component (50). Although CFH is predominantly expressed in the liver compared to other tissues and organs like the muscle, intestine, fins, eyes, and gills (47), present data indicate that the skin may also play a relevant role in the regulation of the alternative pathway of complement in skin secretions. This data is in agreement with different studies that have identified several complement factors in fish skin mucus secretions for several species (30, 51), including gilthead seabream (23, 24).

Our study also revealed that Cathepsin A (*ctsa*) was up-regulated in the skin of fish fed the phytogenic-supplemented diet. An increase in turbot (*Scophthalmus maximus*) *ctsa* expression in skin was described in response to infection challenges, while *ctsa* genes microbial binding capacity was also observed, in which a high affinity to LPS, and a lower affinity to lipoteichoic acid (LTA) and peptidoglycan (PGN), was suggested to be implicated in the sensing and phagocytosis of bacterial pathogens (52). By contrast, in our study the expression of Cathepsin L (*ctsl*) was down-regulated. Different cathepsins have been detected in the mucus of several fish species, which were observed to exhibit high bacteriolytic activity against several fish pathogens (53, 54), evidencing their key role in fish mucosal innate immunity. Fish skin *ctsl* transcripts were observed to be significantly up-regulated after challenges with several bacterial pathogens, including *V. anguillarum* (54, 55). Moreover, as for *ctsa* genes, it was demonstrated that *ctsl* genes have strong

in vitro binding capacity to microbial ligands, suggesting an important role of *ctsl* in fish mucosal immunity (55). The different gene expression pattern observed for *ctsa* and *ctsl* could be related to a time-dependent response, as suggested previously for genes involved in the immune response in fish subjected to feeding trials (56). The differential expression between both cathepsins could be also attributed to the mucosal tissue response specificity. In this way, it has been reported that the same stimulus may differentially modulate the expression for the same genes depending of the mucosal tissue evaluated (57). However, if both genes are linked with the decrease in the bacterial growth observed at skin mucus deserves further investigations.

Fish professional phagocytes include macrophages, granulocytes, dendritic cells and B cells, and as for other vertebrates, phagocytosis in fish is recognized as a critical component of the innate and adaptive immune responses against pathogens, known to elicit several antimicrobial mechanisms. Under this context, the KEGG "Phagosome" pathway obtained from our functional analysis suggests the modulation of phagocytic events by the administered phytogenics. For instance, despite the extracellular roles of cathepsins (58), these proteins are mainly found in endolysosomal structures where they are crucial for protein degradation and Major Histocompatibility Complex (MHC) Class II mediated immune responses (59). Interestingly, the MHC Class II Alpha gene (*mhc-IIa*) was the second most up-regulated gene in the skin of fish fed the phytogenic-supplemented diet. While MHC-IIa protein was identified in gilthead seabream skin mucus proteome (34), the main function of fish MHC Class II molecules is to present the peptides generated in the endolysosomal structure on the cell surface of B cells and phagocytes for their recognition by the CD4+ T cells (60). In fact, the gilthead seabream acidophilic granulocytes, considered the main professional phagocytic cell type for this fish species, were demonstrated to show high *mhc-IIa* gene expression (61). Moreover, they have also proved to have phagocytic activity against bacterial pathogens such *V. anguillarum* (62), being able to release antimicrobial peptides into the phagosome of the ingested pathogenic bacteria (63). The "leukocyte activation", "regulation of T cell proliferation", and "antigen processing and presentation of exogenous peptide antigen via MHC class I" biological processes obtained from our enrichment analysis, might suggest the activity of acidophilic granulocytes and/or other immune cells in the skin of gilthead seabream fed the functional diet. Similarly, previous transcriptional results on the effect of the same functional feed additive in gilthead seabream mucosal tissues such gills (17) suggested the recruitment and activation of acidophilic granulocytes as a consequence of the immunostimulatory effect of this additive.

The functionality and modulation of genes related to the "endosome" and "lysosome" cellular components reinforce the hypothesis of an increased professional phagocytic activity in the skin of fish fed the phytogenic-supplemented diet. Among them, Rab-interacting proteins coding genes (*rilp*, *rab11fip1*) were up-regulated. These genes are involved in several processes like (i) endosomal recycling (64), (ii) endocytic transport to degradative compartments (65) and (iii) in the control of membrane trafficking along the phagocytic pathway (66). In addition, the Microtubule Associated Protein 1 Light Chain 3 Gamma (*map1lc3c*) was up-regulated. This autophagy-related protein is involved in the LC3-associated phagocytosis, in which LC3 is recruited to the phagosome membrane during phagocytosis of pathogens, enhancing the fusion between phagosome and lysosomes (67). In fact, fish epidermal macrophages are characterized by well-developed endoplasmic reticulum and Golgi areas and several lysosome-like vesicles and phagosomes (68). Remarkably, the up-regulation of the MAF BZIP Transcription Factor B (*mafb*), a myeloid lineage-specific transcription factor, which expression levels increase during macrophage differentiation and maturation (69), was also observed in the skin of fish fed the phytogenic-supplemented diet. Therefore, the regulation of LC3 proteins-coding genes and the up-regulation of *mafb* by the phytogenics might support the participation of phagocytic cells in the immune response from the skin observed in our study. The down-regulation of Microtubule Associated Protein 1 Light Chain 3 Beta (*map1lc3b*) opens the possibility to selective and differential mechanisms of activation aimed to the promotion of the phagocytic activity in response to phytogenic supplementation.

Garlic, carvacrol, and/or thymol have been several times described to improve immune cells phagocytic capacity. For instance, dietary garlic (0.5 and 1% inclusion) enhanced the activity of head kidney macrophages phagocytic in rainbow trout (*Oncorhynchus mykiss*) (70). Similar results were observed for blood leukocytes of juvenile hybrid tilapia (*Oreochromis niloticus* x *Oreochromis aureus*) fed a 0.5% garlic-supplemented diet (71). Likewise, carvacrol and thymol supplementation (0.2%) in juvenile hybrid tilapia's diet significantly enhanced phagocytosis of head kidney macrophages (72). The phagocytic activity of serum leukocytes from common carp (*Cyprinus carpio*) was increased due to dietary oregano's essential oil, which is rich in carvacrol and thymol, in a dose-dependent manner (73). A similar enhanced head kidney leukocytes' phagocytosis was also found in gilthead seabream fed a diet supplemented with oregano powder (0.5 and 1%) (74).

Phagocytic events are driven by rearrangements of the actin cytoskeleton (75). Calponin 2 (*cnn2*), an actin cytoskeleton-associated regulatory protein that restricts the pro-inflammatory activation of macrophages (76), was up-regulated in the skin by the phytogenics. Similarly, the Actin Beta (*actb*) gene was also up-regulated. Actin is commonly found in the mucus of several fish species, including gilthead seabream (23, 32), which has led to speculations on its immune function in fish defense. In fact, ACTB levels were observed to be significantly increased in sea lice challenged Atlantic salmon (30, 51). In gilthead seabream, *actb* expression in skin mucus was favored by a dietary probiotic administration (24). Furthermore, the extracellular cytoplasmic actin in insects was observed to bind to bacteria surface, mediating its phagocytosis and killing (77), suggesting that actin could be functionally active in fish skin mucus as well. However, this hypothesis needs further investigation. In summary, our transcriptional analysis could be indicating an enhanced phagocyte function in the skin of fish fed the

phytogenic-diet, which would suggest the promotion of the host's defense ability in resisting bacterial infections.

In order to prevent the stable colonization of potential pathogens, mucus is continuously synthesized, secreted and replaced (26). This continuous regulation of mucus secretions represents one of the first barriers against potential pathogens and toxins (10). Accordingly, our functional analysis determined the modulation of the KEGG "Protein processing in endoplasmic reticulum" pathway and several cellular components connected to the secretory pathway, such as transport vesicles, Golgi-associated vesicles and vacuoles. The regulation of the secretory machinery could be supporting the active biosynthesis and release of immune-related factors on the skin mucus that would mediate in the response observed in our study. The mucus secretion is a complex process that represents the endpoint of the interaction between the innate immune system, endocytosis and autophagy events, ROS generation and mucin secretion (78). From the immunological point of view, cytokines, and chemokines are molecules trafficked in secretory granules and vesicles through secretory pathways in immune cells (79) that could be also involved in the response observed. Under this context, transcripts of the pro-inflammatory cytokine Interleukin 1 Beta (*il-1β*) were observed to be increased in the skin of fish fed the phytogenic-supplemented diet. In fish, IL-1β is a recognized chemoattractant for leukocytes (80). Additionally, the Aminoacyl tRNA Synthetase Complex Interacting Multifunctional Protein 1 (*aimp1*) gene was also up-regulated. Secreted AIMP1 possesses inflammatory cytokine activity responsible for activating monocytes and inducing the production of pro-inflammatory cytokines, mainly the Tumor Necrosis Factor (TNF) (81). In accordance, genes that are activated in response to the pro-inflammatory cytokine TNF, such the TNF Alpha Induced Protein 2 (*tnfaip2*) and TNFAIP3-Interacting Protein 2 (*tnip2*) were also up-regulated by the phytogenics in our study. The last inhibits the NF-kB pathway activation (82), negatively regulating the transcription of other pro-inflammatory cytokines and, consequently, controlling the inflammatory response. Thus, such response could be intimately related with the role of AIMP1 in dermal fibroblast proliferation and wound repair (83).

Other mediators of the inflammatory response were also observed to be regulated in the skin of fish fed the phytogenic-diet. For instance, the Negative Regulator of Reactive Oxygen Species (*nrros*) gene expression was increased. The NRROS protein regulates ROS production by phagocytes during inflammatory response, allowing phagocytes to produce high amounts of ROS in case of infection, while minimizing host's tissue damage (84). NRROS is also suggested to play a role in the maintenance of the immune homeostasis through the inhibition of TLR-mediated MAPK and NF-kB activation (85). Another negative mediator of the inflammatory response, the NLR Family CARD Domain Containing 3 (*nlrc3*) was also observed to be up-regulated in fish fed the phytogenic-supplemented diet. NLRC3 is known to negatively regulate NLR-mediated inflammatory responses (86). In several fish species, the overexpression of *nlrc3* was observed to be systematically induced by bacterial and LPS challenges (87, 88), including in mucosal tissues (89), demonstrating its important role in the fish innate immune response and homeostasis maintenance. The DEAD-Box Helicase 1 (*ddx1*), reported to enhance NF-kB mediated transcriptional activation (90) and recently associated to antiviral responses in fish (91), was also down-regulated in the skin of fish fed the phytogenic-supplemented diet. Therefore, according to the overall response observed considering the transcriptomic profiling of *il-1β*, *aimp1*, *tnfaip2*, *tnip2*, *nrros*, *nlrc3*, and *ddx1*, the regulation of these pro- and anti-inflammatory genes suggests an active cytokine secretion, an immune cell-cell signaling, and the tight control of such response.

Regardless of its critical function protecting the host, the skin mucus also represents an important portal of entry for pathogens since it can provide a favorable microenvironment for some bacteria, the main disease agents for fish, which may induce the development of biofilms depending on the pathogen adhesion capacity (10). Interestingly, the down-regulation of genes of the KEGG "*Vibrio cholerae* infection" pathway was obtained from the analysis of the DEGs of fish fed the phytogenic-based additive. Although the analysis was performed using *Homo sapiens* as model organism, the down-regulation of several genes of the "*Vibrio cholerae* infection" KEGG pathway could also be applied, from a comparative point of view, to an infection process involving a *Vibrio* species in a mucosal tissue, such as *V. anguillarum*, as a consequence of the protective effect of the additive. For instance, the down-regulation of several coding genes for V-type H+ ATPase subunits (*atp6v1c1*, *atp6v1b1*, *atp6ap1*) in fish fed the phytogenic-supplemented diet was obtained. The V-type H+ ATPase complex in endosomes and lysosomes is responsible for the vesicle import of protons and maintenance of the internal acidic pH, crucial for degradative enzymes activity (92). Apart from the "proton-transporting two-sector ATPase complex" cellular component obtained from our enrichment analysis, the down-regulation of the above-mentioned genes also figured in the KEGG "Phagosome" pathway, which could be suggesting a decreased phagosomal activity at the sampling time evaluated (65 days of diet administration). Such gene modulation could be associated to a decrease in skin bacterial pathogens in our fish fed with phytogenic-supplemented diet. This hypothesis is in agreement with the results obtained from the bacterial growth assessment in the epidermal mucus *in vitro*.

Bacterial growth in gilthead seabream mucus was evaluated for both dietary groups, control and phytogenic-supplemented diets, using two pathogenic fish bacteria, *V. anguillarum* and *P. anguilliseptica*, as well as a non-pathogenic fish bacterium, *E. coli*. Our study revealed that the tested feed additive reduced bacteria growth capacity, suggesting an enhanced skin mucus inhibitory capacity against both *V. anguillarum* and *P. anguilliseptica*. The chosen pathogenic bacteria are widely recognized as disease causing agents in several fish species (93, 94), including gilthead seabream (95). The observed reduced growth capacity of *V. anguillarum* in the skin mucus of gilthead seabream fed the phytogenic-supplemented diet is especially relevant because of the evidence that *Vibrio* strains exhibit a chemotactic response to mucus (37). In the last years, disease records indicate that *Vibrio* spp. infections are the most common bacterial

infections in gilthead seabream, mostly reported during the hatchery phase (96). Besides, *P. anguilliseptica* is one of the main agents responsible for outbreaks associated with "winter disease" in gilthead seabream farming, being considered a more opportunistic pathogen whose infections in gilthead seabream usually occur when fish are under environmental stress (97). The different growth curves behavior of *V. anguillarum* and *P. anguilliseptica* in gilthead seabream mucus may be attributed to different virulence of pathogenic bacteria, differences in chemotaxis to skin mucus and adherence capacity.

Our results suggest that the phytogenic-supplemented diet may reduce the settlement of the studied bacteria in the skin surface, decreasing the risk of infection. Previous nutritional studies in which diets containing garlic, carvacrol, or thymol provided effective mucus antibacterial characteristics against fish pathogens. For instance, dietary garlic supplementation (5 and 10 g kg-1) was demonstrated to significantly increase skin mucus antimicrobial activity against several bacterial pathogens in the freshwater Caspian roach (*Rutilus rutilus*) (98). A similar increased bactericidal activity against *Photodamselae* subsp. *piscicida* was observed in the skin mucus of gilthead seabream juveniles fed diets supplemented with oregano powder (0.5 and 1% inclusion) for 15 and 30 days (74). In addition, an enhanced skin mucus bactericidal activity against *V. parahaemolyticus* and *Aeromonas hydrophila* was observed in Pacific red snapper (*Lutjanus peru*) fed a diet supplemented with a medicinal plant extract rich in carvacrol and thymol (0.5, 1, and 2% inclusion) (99). On the contrary, other studies incorporating *Oliveria decumbens*, which is rich in carvacrol (18.8 and 52.9% included in an essential oil and aromatic water fractions, respectively) and thymol (20.3 and 37.6% included in an essential oil and an aromatic water fractions, respectively), in Nile tilapia (*O. niloticus*) diets reported the absence of changes in mucus bactericidal activity against *Streptococcus iniae*. By contrast, the compounds showed high antibacterial capacity when evaluated *in vitro* (100). Similarly, Beltrán et al. (74) did not find an enhancement of antibacterial activity against *V. anguillarum* in the mucus of gilthead seabream fed a diet supplemented with *Origanum vulgare* at 0.5 and 1.0%, although a significant increased bactericidal activity against *Photobacterium damselae* subsp. *piscicida* was observed. The above-mentioned contradictory results could be a consequence of different factors, such as different phytogenics and their content in bioactive compounds, supplementation period of the functional diet, dietary compounds delivery and bioavailability, or an evidence of the synergy between the phytogenics tested that might improve such bactericidal capacity.

Moreover, the *E. coli*, a non-pathogenic bacterium for fish, was also used as an indicator of the potential antibacterial capacity of the skin mucus, neglecting a potentially acquired immunization. Although no significant differences were observed among the experimental diets, a decreasing trend in *E. coli* growth was observed due to the presence of mucus from both dietary groups in the culture medium. Interestingly, garlic-supplemented diets caused a significant increase in the Caspian roach fry skin mucus antibacterial activity against *E. coli* when compared to the control group (98). In addition, carvacrol and thymol were reported to have a bacteriostatic and bacteriolytic *in vitro* activity against most Gram positive and negative bacteria, including *E. coli* (101). Nevertheless, the absence of an inhibitory response against *E. coli* growth in our study could be suggesting the promotion of the skin innate immunity against fish pathogens (102).

From a physiological perspective, bacterial pathogens such *V. anguillarum* are able to elicit strong cortisol-mediated stress responses when they adhere to the mucosal surface (36). For this reason, in this current study we also measured classic skin mucus stress biomarkers in order to establish a correlation between the gene expression profile, antibacterial response and the fish physiological status. Therefore, only cortisol was observed to be significantly reduced in fish fed the phytogenic-supplemented diet. Although the exact mechanisms involved in cortisol exudation through fish mucus are still unclear, cortisol is the main glucocorticoid and the final product of the HPI axis response to stress, varying considerably among species and according to the duration and severity of the stressor (103). Cortisol decrease also favors the fish local mucosal immunity, promoting more effective defense responses against pathogens (104). Besides the hypothesized decrease in skin pathogenic bacteria and its potential impact on cortisol-induced responses, and *vice versa*, several phytogenic active substances have been reported to have sedative properties in fish (105). For instance, garlic powder inclusion in common carp diet (0.5, 1, and 1.5%) decreased plasma cortisol and glucose levels, mitigating ammonia stress-induced effects (106). Similar results were obtained in rainbow trout fed 3% garlic powder supplemented diets (107). Accordingly, the dietary supplementation of a similar additive containing garlic and Labiatae plant essential oils (0.02% inclusion) reduced significantly plasma cortisol levels in European seabass (*Dicentrarchus labrax*) challenged by confinement (108). Although the diet effect on fish stress response is usually evaluated in blood, a positive correlation between cortisol levels on plasma and fish mucus was demonstrated (109, 110) including for gilthead seabream (111). Therefore, the observed decrease in mucus cortisol may suggest a decrease in the allostatic load due to the properties of the phytogenics used in this current study and/or as a consequence of the promotion of the non-specific innate immunity, although these two hypotheses are not mutually excluding.

During stress adaptation, cortisol has been suggested as a signal factor that induces tissue specific molecular programming in fish (112). Cortisol is able to induce a skin local stress response in fish (57, 94, 109, 113), which is particularly characterized by the increase of the secretory activity, related vesicles, apoptosis (114), and transcriptional alterations (57). Under this context, the observed changes in mucus cortisol secretion might be contributing to the obtained secretory-related transcriptional response as well. Furthermore, since a correlation between cortisol secretion and skin mucus oxidative stress was demonstrated (115), a reduction of the skin oxidative stress in response of an increase in antioxidative power induced by the tested phytogenics would be also expected. In fact, in our current study genes coding for antioxidative enzymes were observed to be up-regulated in the skin of fish fed the phytogenic-supplemented diet, as for example the Glutathione S-Transferase Kappa 1 (*gstk1*). GSTK1 belongs to the as Glutathione S-Transferase (GST) superfamily of oxidative stress enzymes,

which are mainly known for their important role in cellular detoxification (116). GSTs have been used as markers for fish antioxidative capacity (117), including the evaluation of phytogenics in aquafeeds (118). Under stress-imposed conditions or injury, the transcription of skin *gst* is usually decreased and associated to immunosuppression in gilthead seabream (33). The Nuclear Factor Erythroid 2 Like 2 (*nfe2l2*), an important transcription factor that positively regulates the expression of cytoprotective genes (119), and the N-Acetyltransferase 1 (*nat1*), known for its participation in the detoxification of drugs and other xenobiotics (120), were also observed to be up-regulated by the functional diet. In addition, Heat Shock Protein family genes (*dnajc8*, *dnajb9*, and *hspa14*) were down-regulated in the fish fed the phytogenic-supplemented diet. In particular, HSPA14 is member of the Hsp70 family, which proteins levels have been described to increase in fish under stress or pathological conditions (121, 122). Therefore, the regulation of these genes is supporting the involvement of immune cells in the skin response observed, suggesting a reduction of the skin oxidative stress in response of the reduced mucus cortisol secretion and/or by the antioxidative characteristics of the tested phytogenics. In fact, the inclusion of garlic (106, 123), carvacrol, and thymol (73, 124) in aquafeeds have been continuously demonstrated to enhanced fish antioxidant status. In the present study, the epidermal mucus antioxidant capacity was also measured by mean of the FRAP analysis (102, 115). Although our transcriptional analysis revealed the regulation of several markers of oxidative stress, according to FRAP's analyses the mucus antioxidant power was not significantly changed by the tested additive. Our results are in agreement with some previous studies which reported that the dietary supplementation of carvacrol and/or thymol-rich compounds did not affect the skin mucus biochemical contents or mucus antioxidant status (74, 100).

Collectively, these data clearly suggest a relationship between the tested phytogenics, the increased skin innate immunity and a cortisol-mediated response, promoting the overall animal welfare. In fact, according to the only biological process significantly regulated among the experimental diets, the RNA processing biological process, several genes implicated in ribosomal proteins synthesis (*riok1*, *rcl1*, *rrp1*, *nop56*, *nsa2*, *tsr2*) were mainly up-regulated in fish fed the phytogenic-supplemented diet. Since ribosome biogenesis is the cell's most costly process in terms of energy expenditure, this process must be tightly regulated in order to avoid wasted energy (125). Consequently, the up-regulation of such genes could be suggesting less stressed cells able to direct their energy into this process. Since skin from the control group appear to be more susceptible to be colonized by pathogenic bacterial strains, it could be spending more energy in defense mechanisms and bacterial clearance than fish fed the phytogenic-supplemented diet.

In summary, our analysis of the skin transcriptional profiling as well as the skin mucus biomarkers and lower pathogenic bacterial growth capacity revealed a multifactorial response to the dietary administration of garlic essential oil, carvacrol, and thymol, mainly through the transcriptional regulation of factors of the innate immunity and the stimulation of the secretory pathway. Our results suggest that the phytogenic-supplemented diet induces the activation of the mucosal immune response that promotes the secretion of non-specific immune molecules into the skin mucus, resulting in the decrease of bacterial growth capacity in mucus. From our transcriptomic enrichment analysis, the regulation of genes related with the secretory pathway suggests that the tested phytogenics could be also stimulating the recruitment of phagocytic cells. The reduction in skin mucus cortisol is in line with the recognized properties of the phytogenics. Since the exact mechanisms promoted by the tested phytogenics were not yet demystified, further analysis should be made in order to assess the effect of the experimental diet on skin phagocytes and phagocytosis potential. More efforts are also needed for determining the impact of the functional additive on the skin defensive status against other pathogenic bacteria that threaten the success of aquaculture under intensive farming regime.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

IRTA facilities, where the trial was conducted, are certified and have the necessary authorization for the breeding and husbandry of animals for scientific purposes. All procedures involving the handling and treatment of the fish were approved as far as the care and use of experimental animals are concerned, by the European Union (86/609/EU), the Spanish Government (RD 1201/2005), and the University of Barcelona (Spain).

AUTHOR CONTRIBUTIONS

Biological samplings were performed by EG, JF, RS, LF-A, IS, and AI. The transcriptomic data analysis and interpretation was performed by JF, RS, EV-V, and FER-L. LF-A, IS, and AI carried out the skin mucus analyses. The study was supervised by EG, FER-L, and LT. JF wrote the original draft. Funding was obtained by EG and LT. All the authors provided critical feedback, read, and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.633621/full#supplementary-material

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CHAPTER IV

Review: Phytogenic bioactive compounds shape fish mucosal immunity

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REVIEW
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Phytogenic Bioactive Compounds Shape Fish Mucosal Immunity

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Aquaculture growth will unavoidably involve the implementation of innovative and sustainable production strategies, being functional feeds among the most promising ones. A wide spectrum of phytogenics, particularly those containing terpenes and organosulfur compounds, are increasingly studied in aquafeeds, due to their growth promoting, antimicrobial, immunostimulant, antioxidant, anti-inflammatory and sedative properties. This trend relies on the importance of the mucosal barrier in the fish defense. Establishing the phytogenics' mode of action in mucosal tissues is of importance for further use and safe administration. Although the impact of phytogenics upon fish mucosal immunity has been extensively approached, most of the studies fail in addressing the mechanisms underlying their pharmacological effects. Unstandardized testing as an extended practice also questions the reproducibility and safety of such studies, limiting the use of phytogenics at commercial scale. The information presented herein provides insight on the fish mucosal immune responses to phytogenics, suggesting their mode of action, and ultimately encouraging the practice of reliable and reproducible research for novel feed additives for aquafeeds. For proper screening, characterization and optimization of their mode of action, we encourage the evaluation of purified compounds using *in vitro* systems before moving forward to *in vivo* trials. The formulation of additives with combinations of compounds previously characterized is recommended to avoid bacterial resistance. To improve the delivery of phytogenics and overcome limitations associated to compounds volatility and susceptibility to degradation, the use of encapsulation is advisable. Besides, newer approaches and dedicated methodologies are needed to elucidate the phytogenics pharmacokinetics and mode of action in depth.

Keywords: immunity, MALT, organosulfurs, terpenes, sustainable aquaculture, teleost, phytogenic additive

INTRODUCTION

Sustainable food supply to feed the demand of the projected world population by 2050 is a challenge, in which aquaculture is predicted to be the main source of aquatic dietary proteins. Such growth will unavoidably involve the implementation of innovative aquaculture production strategies, targeting issues related to effective health management and animal welfare (1). In this regard, the development and application of functional feeds represent a sound strategy to improve aquaculture systems, since they provide functional health benefits to animals beyond their nutritional value (2). In this scenario, phytogenics, also known as phytobiotics, are defined as environmentally friendly plant-derived bioactive compounds used as functional feed additives that show positive effects on animal growth and health. Phytogenic often comprise aromatic plants extracts, and essential oils characterized by its richness in biologically active compounds (3, 4). In farmed fish, a wide spectrum of phytogenics have been increasingly studied mainly due to their wide repertoire of properties, including growth promotion, and antimicrobial, immunostimulant, antioxidant, anti-inflammatory and sedative activities (5). In particular, phytogenics derived from Lamiaceae family and *Allium* sp. are among the most commonly studied and administrated plant-based additives (6, 7). Nonetheless, the complexity of the mechanisms of action and the pharmacological effects promoted by the diverse bioactive compounds present in such plants, along with their frequently observed synergistic behavior (8), often limits the full understanding of their biological activity (9).

Since outbreaks of fish diseases are one of the main constrains for the progress of the aquaculture sector (10), the inclusion of phytogenics' in aquafeeds is achieving significant attention at a global scale. The impact of phytogenics upon fish systemic immunity has been extensively tested in the past (5, 9). However, an increasing trend to evaluate phytogenics' impact upon the mucosal immunity has been gained importance in recent years, which is mainly attributed to the importance of the mucosal barrier in the fish defense against variated threats and, potential colonization and invasion by pathogenic organisms (11). In contrast, most of the studies evaluating the effect of phytogenics in fish systemic immunity are only supported by a selection of repetitive primary analyses (*i.e.*, lysozyme, bacteriolytic and complement activities, immunoglobulins, oxidative stress enzymes, etc.) serving only as proxies, that only provide a snapshot of the effects of the evaluated feed additive on the organism. These approaches do not allow elucidating their mode of action at cellular and molecular levels. This is of special relevance when dealing with functional feed additives with potential pharmacological properties, as their standardized use mainly depends on the proper understanding of their regulatory properties in the immune system.

Phytogenic administration has the potential to regulate the mucosal barrier function by means of several molecular mechanisms, in which the phytogenic bioactive compounds interact with cellular transcription factors and metabolic cascades. Therefore, the modulation of the expression of genes coding for immune relevant molecules alter the mucosal protective characteristics and their immunological status (12–16). Besides, the immune system influences the regulation and composition of the microbiota and *vice versa*, an interaction that plays a determinant role in the maintenance of the mucosal integrity and functionality (17, 18). Hence, both the improvement of the mucosal barrier characteristics and the modulation of the microbiota are central targets for the development of new phytogenic additives, while understanding their mode of action at cellular and molecular levels is critical for elucidating their benefits to the host.

Given the extended literature available on plant-based functional additives and the significance of the mucosal immunity described above, our review efforts focus exclusively on the physiological and immunological responses achieved by the most studied fish mucosal tissues, intestine, gills and skin, of organisms fed with phytogenics of the Lamiaceae family and *Allium* sp. In the first part, we present a thorough description on their main bioactive compounds and relevant biological properties. Then, the immunomodulatory properties and the mechanisms they can trigger on the fish mucosal tissues are explored and further potential mechanisms hypothesized. Finally, research gaps and constrains for the development of applicable phytogenic-based additives are discussed. Overall, the information presented herein aims to provide clear insights on the fish mucosal immune response dietary treated with phytogenics, propose viable mechanisms for exploiting them, and ultimately encourage the practice of reliable and reproducible research for the development of novel feed additives to be used as sustainable and safe prophylactic strategies for aquaculture.

FISH MUCOSAL IMMUNITY AT A GLANCE

The mucosal barrier constitutes the fish first line of defense against the surrounding environment and potential pathogens. Fish mucosal tissues are particularly characterized by a mucosa-associated lymphoid tissue (MALT), harboring diverse myeloid and lymphoid cells that are responsible for the host protection against pathogens and antigens, while tolerating beneficial symbiont colonization to maintain mucosa homeostasis (19–21). Six different MALTs have been described so far in teleosts. The gut-associated lymphoid tissue (GALT), the gill-associated lymphoid tissue (GIALT), the skin-associated lymphoid tissue (SALT), the nasopharynx-associated lymphoid tissue (NALT) and, the more recently characterized the buccal, and pharyngeal MALTs (22). Other mucosal immune systems have been hypothesized and are currently under study (23). Despite the existence of others, the GALT, GIALT and SALT are the most studied and well characterized MALTs and therefore selected as target in this review.

Among the extensive cell types with immune capacity coexisting in the fish body, upon sensing the presence of pathogenic or commensal microbe-associated molecular pattern (MAMP) a downstream signaling response mediated through pattern recognition receptors (PRRs) immediately takes place. So far, several piscine PRRs have been identified, being the toll-like receptors (TLR), NOD-like receptors (NLR) and retinoic

acid-inducible gene I (RIG-I)-like receptors (RLRs) the best characterized (24, 25). Epithelial and endothelial cells together with the professional phagocytes, represented by macrophages, granulocytes and dendritic cells, are the first responders against MAMPs formerly sensed by PRRs. Phagocytosis contributes to both pathogen clearance and subsequent antigen presentation to other immune cells by the membrane Major Histocompatibility Complex (MHC) class II peptide complex (26). In most, but not all teleost fish, the peptide-MHC II complex activates naive $CD4^+$ T cells expressing antigen-specific T cell receptors (TCR) in their surface. Recognition of this complex stimulates the dedicated $CD4^+$ cells activation and differentiation into T helper cell subsets possessing inflammatory cytokines secreting capacity that further coordinate the adaptive response together with B cells (27, 28). Interestingly, while $CD4^+$ T helper lymphocytes are mainly present in the gut *lamina propria*, the cytotoxic $CD8^+$ cells are the dominant intraepithelial resident immunocytes (29–31).

In fact, both T and B lymphocytes are abundantly present in fish mucosal tissues (32). Interestingly, the phagocytic and bacterial-killing abilities of B cells in fish have been fairly introduced in the past (33). However, their antigen presentation mechanisms mediated by MHC II and costimulatory molecules (CD80/CD86 and CD83) to prime naïve $CD4^+$ T-cells, produce IgM, IgT, and eventually IgD plasmablasts -a major lymphocyte population in the gut, gill and at some extent the skin-, have just been recently addressed (34, 35). The IgT, the teleost specialized mucosal immunoglobulin analogous to mammalian IgA (36), plays a critical role in the clearance of mucosal pathogens and the preservation of microbiota homeostasis through immune exclusion (11). Although extremely important in mucosal defense, not all teleosts present IgT/IgD, which suggests the existence of alternative mucosal immune systems (23).

For instance, the complete IgM and IgT sequences in their membrane and soluble forms have been reported and characterized for the first time in a perciform, the gilthead sea bream (*Sparus aurata*) (37). Interestingly, this study demonstrated that virus and bacteria trigger the mucosal immune response by promoting the activation of IgT in seabream. Although, diets with fish oil replacement by ones from plant origin inhibited the IgT up-regulation upon intestinal parasitic challenge, which was related to a worse disease outcome. These results evidenced that mucosal immunoglobulins can be significantly affected by dietary treatments, which highlights the necessity of testing this response case by case.

Although characterized by common cellular components, immune mediators and immune mechanisms, the different composition, organization and functions of MALTs may vary according to each tissue intrinsic and external environmental factors (38), changes that may be associated to the fish species considered. Besides, the microbiota also stands as a relevant component of the mucosal barrier, displaying an antagonistic behavior against invading "hostile" microorganisms and directly participating in the immune responses through the complex host-microbiota crosstalk at the mucosal interface (17, 18). Therefore, the selective manipulation of the microbiota by means of nutritional approaches has been previously proposed as a viable alternative to modulate mucosal responses (39).

The mucosal tissues are intrinsically characterized by mucus secreting cells, such as goblet cells (40). Beside playing important roles in intra- and interspecific ecological interactions (41) and being a key component that ensures host-microbiota homeostasis (42, 43), the secreted semipermeable mucus represents the first challenge that pathogens have to overcome in order to interact with the host. Its complex composition encompasses a matrix of glycoproteins, the mucins that confer the mucus its structure, and a wide variety of humoral immune factors, such as lysozymes, complement, lectins, proteolytic enzymes, antimicrobial peptides, immunoglobulins, among others (41). Moreover, the mucus is continuously secreted and replaced (44); this continuous secretion aims to prevent pathogen attachment and interaction with the host. Therefore, the presence of a mucus layer is fundamentally involved in the regulation of the mucosal immune system, not only as a protective physical and chemical barrier, but also acting as a vehicle for mucins and humoral immune factors from the inside out. Both goblet cells (13, 15) and mucus composition (45) are highly susceptible to be manipulated through dietary strategies, which opens a wide range of possibilities when to design and apply new functional feed additives.

LAMIACEAE FAMILY AND *ALLIUM* SP. AMONG THE MOST STUDIED AROMATIC PLANTS USED AS PHYTOGENICS IN AQUACULTURE

In nature, plant secondary metabolites have functional roles independent from plant growth and development; thus, protecting plants from herbivore and pests, or acting as chemoattractants for pollinators (46). These bioactive compounds broadly found in aromatic plants are usually present as mixtures, mainly represented by phenolics and terpenes that are chemically characterized by their aromatic rings (3). Therefore, their benefits as dietary supplements are subject to the variability and complexity of the aromatic compounds mixture, apart from their synergistic effect, their origin, the dietary inclusion level and their pharmokinetics (47).

In particular, phytogenics derived from Lamiaceae family and *Allium* sp. are among the most widespread administrated plant-based additives in aquaculture (48) and livestock (6, 7). These compounds are used for their recognized growth promoting, antimicrobial, immunostimulant, antioxidant, anti-inflammatory and sedative properties. Although they can be found worldwide, some representatives of this group of aromatic plants (*i.e.*, oregano, thyme, basil, menthe, rosemary, sage, marjoram, garlic and onion, among others) are particularly present and traditionally consumed in the Mediterranean area and appreciated in terms of human nutrition and therapy (49, 50). The health-promoting properties of these aromatic plants have been extensively reviewed in different aquaculture species (5, 51–56). However, most of the studies dealing with these functional feed additives were only focused in

physiological or biochemical responses, but few of them have elucidated the cellular and molecular mechanisms underlying their immunostimulatory capacity.

While the existent information about the inherent effect of these phytogenics upon immune cells is limited under *in vitro* conditions, numerous *in vivo* studies have demonstrated an improvement of the fish mucosal immune responses following their administration. A refined complementary search through Web of Science, PubMed and Google Scholar was performed in this review. Until March 2021, 62 publications reporting the nutritional effects of Lamiaceae family and *Allium* sp., or related bioactive compounds, upon fish mucosal responses were retrieved and their results summarized in **Table 1**. Importantly, most of them were published in the last year; thus, evidencing the current increasing interest for research on phytogenics targeting mucosal tissues. From the overall bibliographic search results, few publications felt within the objective of the present review and described the cellular or molecular mechanisms underlying fish mucosal immune responses to phytogenics' administration. Studies reporting the application of plant extracts or related compounds as bath treatments or evaluating bactericidal or antiparasitic effects *in vitro* were excluded from the selection as this review is just focused on the mucosal immune mechanisms. Furthermore, **Table 1** omits those results out of the mucosal immunity context, including systemic immunity-related results, non-immune digestive parameters or other complementary analysis performed within each study. Although such variables are extensively used as key performance indicators in such studies, their relevance in terms of supporting and/or establishing the mode of action of phytogenics is questionable and out of the scope for this review. Finally, blends with other components besides the selected group of plants –or associated bioactive compounds such as terpenes or organosulfurs– were excluded as well.

Effect of Dietary Terpene Phenolic Compounds Upon Fish Mucosal Immunity

Phenolics and terpenes are a group of volatile plant-derived bioactive compounds with medicinal and biotechnological value that constitute the dominant fraction of the essential oils derived from aromatic plants (3). The monoterpenes carvacrol and its isomer thymol are the most studied phenolic compounds, representing the major components of the essential oils from several aromatic plants of the Lamiaceae family like the oregano (*Origanum vulgare*) and thyme (*Thymus vulgaris*) (110, 111). These compounds are particularly studied and recognized for their bactericidal activity, since their lipophilic character act as bacterial membrane permeabilizers with cytotoxic effects upon bacterial structure and function, leading to membrane expansion, fluidity and permeability, disturbance of the membrane-embedded proteins, respiration inhibition and alteration of ion transport. In addition, carvacrol and thymol were demonstrated to act as quorum sensing (QS) inhibitors, reducing bacterial biofilm formation. Carvacrol in particular, is able to inhibit bacteria motility, collapsing the proton-motive force, depleting the ATP pools and preventing the synthesis of flagellin (112). This bactericidal property highlights the ability of these compounds to potentially modulate mucosal tissues associated microbiota.

Together with their well-studied bactericidal potential, these phenolic compounds are described to potentially improve the integrity of the mucosal tissues due to their observed antioxidant, anti-inflammatory and consequent immunomodulatory properties in the gastrointestinal mucosa of several animal models (113). The reported strong antioxidant activity of carvacrol and thymol rely on their ability to scavenge free radicals, inhibiting reactive oxygen species (ROS) and other oxygen radicals generated in cells and tissues (114). By contrast, high concentrations may display antagonistic pro-oxidant effects (114). This dose-dependent antagonistic activity evidences the importance of correctly define their administration doses in order to obtain the desired results with regard to their immunomodulatory properties.

Regarding their anti-inflammatory potential, carvacrol and thymol appear to interfere with the NF-*k*B and MAPK pathways, modulating the expression of pro-inflammatory and anti-inflammatory cytokines (115, 116). It is commonly speculated that the anti-inflammatory properties of plant-derived bioactive compounds, such as carvacrol and thymol, may be attributed to their capacity to inhibit TLR-mediated NF-*k*B signaling pathways (117, 118). Furthermore, evidence that both carvacrol and thymol play a role in the chemosensory system through the activation of transient receptor potential (TRP) cation channels exist (119, 120). In higher vertebrates, TRP channels are widely expressed in several cellular types that includes most of the mucosal components. Through the maintenance of the intracellular calcium homeostasis, these channels are known to regulate several cell functions, such as stimuli perception, inflammatory molecules production and secretion, migration and even phagocytosis (121–123). Carvacrol and thymol are known to activate both the receptor TRPA1 (120) and the receptor TRPV3 in mucosal tissues, elevating cytosolic Ca^{2+} concentration in epithelial cells (119, 124). In fish, together with TLRs, the activation of TRP channels has been demonstrated to modulate the inflammatory processes through the activation of the TRP/Ca^{2+}/TAK1/NF-*k*B signaling pathway (125). This suggests that a TRP channel mediated cellular activation may underlie the immunomodulatory properties of these bioactive compounds.

The health promoting effects of oregano, thyme and their derivates in fish have been recently reviewed (55, 126). Concerning their impact upon fish mucosal immunity, several nutritional studies have described beneficial effects of phytogenics derived from oregano, thyme and other plants of the Lamiaceae family upon the mucosal tissues in several fish species (**Table 1**). Most of them have reported an increase in skin mucus immune markers and/or skin mucus bactericidal activity (57–59, 66, 72, 74–80). The repeatedly evaluated markers were lysozyme, alkaline phosphatase, complement and protease activities, total immunoglobulin and protein content in fish skin mucus, as well as its *in vitro* bactericidal potential against bacterial pathogens. Several of these studies also described an improvement in key performance indicators, such growth, feed efficiency and survival against pathogenic bacterial challenges

TABLE 1 | Extended summary of the current available literature on nutritional effects of Lamiaceae family and *Allium* sp. derived phytogenics upon fish mucosal immune response.

Phytogenic plant origin	Supplemented form	Inclusion dosage(s) tested	Period of administration	Main bioactive components (≤ 3)	Fish species	Performance	Mucosal parameters evaluation	Key benefits summary	Reference
Lamiaceae family									
Oregano (*Origanum vulgare*)	Powder	0.5%, 1.0% and 2.0%	8 weeks	N/I	Zebrafish (*Danio rerio*)	↑ Final weight ↑ Weight gain ↓ FCR ↑ SGR ↑ Survival against *A. hydrophila*	↑ Skin mucus lysozyme activity ↑ Skin mucus alkaline phosphatase activity ↑ Skin mucus total Ig ↑ Skin mucus protease activity ↑ Skin mucus total protein	Beneficially affects the skin mucus immune parameters, growth performance and survival against pathogenic bacterial challenge	Rashidian, Boldaji (57)
Oregano (*Origanum vulgare*)	Powder	0.5% and 1.0%	15 and 30 days	N/I	Gilthead seabream (*Sparus aurata*)	No effect	↑ Skin mucus IgM ↑ Skin mucus bactericidal activity against *P. damselae*	Oregano improves humoral immunity and increases the antibacterial activity of skin mucus	Beltrán, Gonzalez Silvera (58)
Oregano (*Origanum vulgare*)	Ethanolic extract	0.2% and 0.5%	60 days + 7 days *A. hydrophila* challenge	N/I	Nile tilapia (*Oreochromis niloticus*)	↑ Final weight ↑ Weight gain ↓ FCR ↑ SGR ↑ Survival rate ↑ Survival against *A. hydrophila*	↑ Skin mucus total Ig	Can effectively improve the fish growth, health, and immune status	Mohammadi, Rafiee (59)
Oregano (*Origanum vulgare*)	Powder	5.0, 10.0, 15.0 and 20.0 g kg^{-1}	8 weeks	Carvacrol and thymol (Commercial product)	Common carp (*Cyprinus carpio*)	↑ Final weight ↑ Weight gain ↑ SGR	↑ Intestine villus height ↑ Intestine villus width ↑ Intestine crypt depth	Dose-dependent enhancement of intestinal morphometry, which subsequently lead to improvement of nutrients absorption	Abdel-Latif, Abdel-Tawwab (60)
Oregano (*Origanum vulgare*)	Essential oils	0.75, 1.5, 2.25 and 3.0 g kg^{-1}	64 days	Carvacrol, thymol and p-cymene	Nile tilapia (*Oreochromis niloticus*)	No effect	↑ Intestine villus height	Increases intestinal villus size	Heluy, Ramos (61)
Oregano (*Origanum vulgare*)	Essential oils	0.5, 1.5 and 4.5 g kg^{-1}	8 weeks + 7 days *A. hydrophila* challenge	N/I	Koi carp (*Cyprinus carpio*)	↑ Survival against *A. hydrophila*	↓ TNF-a and TGF-b gene expression in intestine ↑ Actinobacteria phylum, and *Propionibacterium*, *Brevinema* and *Corynebacterium* genera ↓ Bacteroidetes phylum and *Vibrio* genus No effect on bacterial alpha diversity	Presents immunomodulatory effects and enhances disease resistance. Also beneficially alters the gut bacterial community composition of fish	Zhang, Wang (62)
Oregano (*Origanum vulgare*)	Powder	0.5% and 1.0%	30 days + cypermethrin exposure	Carvacrol and thymol (Commercial product)	Common carp (*Cyprinus carpio*)	N/I	↓ Gill histopathologic lesions ↓ Gill proliferating cell nuclear antigen (PCNA) and caspase-3 immune positive cells	Protective roles against the adverse effects of cypermethrin, enhancing recovery from the exposure	Khafaga, Naiel (63)
Oregano (*Origanum vulgare*)	Essential oils	0.01%, 0.02%, 0.05% and 0.10%	24 days + 28 days *I. salmonis* and *T. truttae* challenge (Total 52 days)	Carvacrol, p-cymene and γ-terpinene	Chum salmon (*Oncorhynchus keta*)	↑ feed efficiency ↓ *I. salmonis* infection ↓ *T. truttae* infection ↓ cumulative mortality	Carvacrol content detected in the skin of fish fed the oregano supplemented diet	Preventive effects against *I. salmonis* and *T. truttae* and suggests the possibility that its anti-parasitic action is attributable to the bioactive component emergence through the skin	Mizuno, Urawa (64)
Oregano (*Origanum vulgare*)	Essential oils	0.5, 1.0, 1.5, 2.0 and 2.5 g kg^{-1}	90 days	N/I (Commercial product)	Yellow tail tetra (*Astyanax altiparanae*)	N/I	↑ Intestine villus length ↑ Intestine villus width ↑ Intestine absorption area ↑↓ Intestine goblet cells number	Promotes increased absorption surface area and modulates the number of goblet cells involved in protecting the intestinal mucosa	Ferreira, Caldas (65)
Marjoram (*Origanum majorana*)	Ethanolic extract	0.1, 0.2 and 0.4 g kg^{-1}	60 days + 10 days *A. hydrophila* challenge	N/I	Common carp (*Cyprinus carpio*)	↑ Final weight ↑ Weight gain ↓ FCR ↑ SGR ↑ Survival against *A. hydrophila*	↑ Skin mucus alkaline phosphatase ↑ Skin mucus total Ig ↑ Skin mucus lysozyme activity ↑ Skin mucus alternative complement (ACH50) activity	Increase fish skin mucosal immunity and performance	Yousefi, Ghafarifarsani (66)
Thyme (*Thymus vulgaris*)	Essential oils	500 ppm	30 days + thiamethoxam exposure	Thymol, p-cymene and γ-terpinene	African catfish (*Clarias garipenus*)	N/I	↓ Gill histopathologic lesions	Mitigate the thiamethoxam induced toxicity	El Euony, Elblehi (67)
Thyme (*Thymus vulgaris*)	Aqueous extract	5.0, 10.0 and 20.0 g kg^{-1}	2 weeks + oxytetracycline	N/I	Rainbow trout (*Oncorhynchus mykiss*)	No effect	↑ Intestine antioxidant enzymes (SOD, CAT, GPx and GST) activity ↓ Intestine levels of the oxidative stress marker malondialdehyde	Mitigate adverse effects of oxytetracycline and improve the fish immune responses	Hoseini and Yousefi (68)

(Continued)

TABLE 1 | Continued

Phytogenic plant origin	Supplemented form	Inclusion dosage(s) tested	Period of administration	Main bioactive components (≤ 3)	Fish species	Performance	Mucosal parameters evaluation	Key benefits summary	Reference
Thyme (*Thymus vulgaris*)	Essential oils	0.1%, 0.5%, and 1%	15 days	Thymol, o-cymene and carvacrol	Nile tilapia (*Oreochromis niloticus*)	N/I	No effect upon the population of beneficial Bacillus bacteria in the gut	Stimulated the cellular components of the non-specific immune response without deleterious effects on the general health of the fish or the intestinal tract	Valladão, Gallani (69)
Thyme (*Thymus vulgaris*)	Essential oils	0.005, 0.010 and 0.02 g kg^{-1}	5 weeks	Thymol, p-cymene and linalool	Rainbow trout (*Oncorhynchus mykiss*)	No effect	No effect upon the allochthonous microbiota profile	No toxic effects do not significantly alter the intestinal contents bacterial populations	Navarrete, Toledo (70)
Spanish thyme (*Thymus zygis* subsp. *gracilis*)	Essential oils	0.001, 0.002, 0.003 and 0.004 g kg^{-1}	12 weeks	Thymol, p-cymene and carvacrol	Gilthead seabream (*Sparus aurata*)	No effect	↑ Anterior intestine lymphocyte aggregates in the lamina propria at low dose ↓ Anterior intestine lymphocyte aggregates in the lamina propria at high doses	Dose-dependent immuno-modulatory effect upon the intestine	Hernandez, Garcia (71)
Lemon balm (*Melissa officinalis*)	Ethanolic extract	0.2% and 0.5%	60 days + 7 days *A. hydrophila* challenge	N/I	Nile tilapia (*Oreochromis niloticus*)	↑ Final weight ↑ Weight gain ↓ FCR ↑ SGR ↑ Survival rate ↑ Survival against *A. hydrophila*	↑ Skin mucus total Ig ↑ Skin mucus lysozyme activity ↑ Skin mucus protease activity ↑ Skin mucus alternative complement (ACH50) activity	Can effectively improve the fish growth, health, and immune status	Mohammadi, Rafiee (59)
Peppermint (*Mentha piperita*)	Powder	2.0, 3.0, and 4.0 g kg^{-1}	8 weeks	N/I	Caspian roach (*Rutilus caspicus*)	↑ Final weight ↑ Weight gain ↓ FCR ↑ SGR ↓ Daily intake rate	↑ Secretion of skin mucosal protein pattern bands; higher lysozyme band intensity in particular ↑ Skin mucus lysozyme activity ↑ Skin mucus alkaline phosphatase activity ↑ Skin mucus soluble protein	Act as a growth promoter and immunostimulant	Paknejad, Hosseini Shekarabi (72)
Peppermint (*Mentha piperita*)	Essential oils	0.1 and 0.25 g kg^{-1}	7, 14, 30 and 60 days	Menthol, mentone and 1,8-cineole	Nile tilapia (*Oreochromis niloticus*)	N/I	↑ Intestine intraepithelial lymphocytes	Show benefits in terms of intestinal health and on immune parameters	Valladão, Gallani (73)
Peppermint (*Mentha piperita*)	Ethanolic extract	1.0%, 2.0% and 3.0%	8 weeks	N/I	Rainbow trout (*Onchorhynchus mykiss*)	↑ Survival against *A. hydrophila*	↑ Skin mucus antibacterial activity against *S. iniae*, *Y. ruckeri*, *A. hydrophila* and *L. garvieae*	Triggers the immune system of rainbow trout against *Y. ruckeri*	Adel, Pourgholam (74)
Peppermint (*Mentha piperita*)	Ethanolic extract	1.0%, 2.0% and 3.0%	56 days	N/I	Caspian kutum roach (*Rutilus frisii kutum*)	↑ Weight gain ↓ FCR ↑ SGR	↑ Skin mucus antibacterial activity against *S. iniae*, *Y. ruckeri*, *L. monocytogenes* and *E. coli* ↑ Skin mucus protein level ↑ Skin mucus alkaline phosphatase activity	Increases the mucosal immune parameters and performance of fry in a dose dependent manner	Adel, Amiri (75)
Peppermint (*Mentha piperita*)	Ethanolic extract	1.0%, 2.0% and 3.0%	8 weeks	N/I	Caspian brown trout (*Salmo trutta caspius*)	↑ Weight gain ↓ FCR ↑ SGR	↑ Skin mucus protein level ↑ Skin mucus lysozyme activity ↑ Skin mucus alkaline phosphatase activity	Promote growth performance and have immunostimulant properties	Adel, Safari (76)
Horsemint (*Mentha longifolia*)	Ethanolic extract	2.0%, 4.0% and 6.0%	8 weeks + *Y. ruckeri* challenge	N/I	Caspian kutum roach (*Rutilus frisii kutum*)	↑ Weight gain ↓ FCR ↑ SGR ↑ Survival rate	↑ Skin mucus protein level ↑ Skin mucus lysozyme activity ↑ Skin mucus alkaline phosphatase activity ↑ Skin mucus protease activity ↑ Skin mucus esterase activity ↑ Skin mucus antibacterial activity against *S. iniae*, *Y. ruckeri*, *A. hydrophila* and *L. garvieae*	Improve growth performance and boost fish immune response in a dose-related manner	Gholamhosseini, Adel (77)
Horsemint (*Mentha longifolia*)	Hydroalcoholic extract	0.1%, 0.2% and 0.3%	4 weeks + 10 days *Y. ruckeri* challenge	N/I	Rainbow trout (*Onchorhynchus mykiss*)	↑ Survival against *Y. ruckeri*	↑ Secretion of skin mucosal protein pattern bands; higher lysozyme band intensity in particular	Dose-related positive effect on immunogenicity and increased resistance to bacterial disease	Heydari, Firouzbakhsh (78)
Thumbai (*Leucas aspera*)	Powder	1.0, 2.0, 4.0 and 8.0 g kg^{-1}	45 days + 15 days *S. agalactiae* challenge	N/I	Nile tilapia (*Oreochromis niloticus*)	↑ Weight gain ↓ FCR ↑ SGR ↑ Survival	↑ Skin mucus lysozyme activity ↑ Skin mucus peroxidase activity	Increase skin mucosal immune parameters, performance and survival against bacterial infection	Kurian, Van Doan (79)

(Continued)

TABLE 1 | Continued

Phytogenic plant origin	Supplemented form	Inclusion dosage(s) tested	Period of administration	Main bioactive components (≤ 3)	Fish species	Performance	Mucosal parameters evaluation	Key benefits summary	Reference
						against *S. agalactiae*			
Shirazi thyme (*Zataria multiflora*)	Hydroalcoholic extract	2.0 g kg^{-1}	56 days	Thymol and carvacrol? (N/I)	Rainbow trout (*Oncorhynchus mykiss*)	↑ Survival rate	↑ Skin mucus bactericidal activity against *A. hydrophila* ↑ Skin mucus lysozyme activity	Increase skin mucosal immunity	Mirghaed, Hoseini (80)
Shirazi thyme (*Zataria multiflora*) + Rosemary (*Rosmarinus officinalis*)	Powder (1:1) + aflatoxin B1	40 g kg^{-1} (20 g kg-1 each)	12 weeks	N/I	Common carp (*Cyprinus carpio*)	No effect	No effect	Do not prevent intestinal tissue lesions induced by aflatoxin B1	Tasa, Imani (81)
Rosemary (*Rosmarinus officinalis*)	Aqueous extract	10, 20, 40, 80 and 100 ml/ 100 g	20 days	1,8-Cineole	Common carp (*Cyprinus carpio*)	N/I	↑ Skin mucus level of 1,8-Cineole dose-dependent No effect upon intestine histopathology	High volume of extracts might promote hepatic toxicity	Zoral, Ishikawa (82)
Rosemary (*Rosmarinus officinalis*)	Powder	0.6, 1.2, 1.8 and 2.4 g kg^{-1}	4 and 12 weeks	Carnosic acid and carnosol (1:1)	Gilthead seabream (*Sparus aurata*)	No effect	No effect	The histological examination of the intestine showed no aspects that might pose problems for absorption, or any immune system disorder associated with the intestine	Hernandez, Garcia Garcia (83)
Oliveria (*Oliveria decumbens*)	Essential oils and/ or hydroethanolic extract	0.01%, 0.1% and 1.0%	60 days + 14 days *S. iniae* challenge	γ-terpinene, carvacrol and thymol	Nile tilapia (*Oreochromis niloticus*)	↑↓ Survival against *S. iniae*	No effect	Increase fish survival 14 days after challenge with *S. iniae*	Vazirzadeh, Jalali (84)
Clove basil (*Ocimum gratissimum*)	Ethanolic extract	5.0, 10.0, and 15.0 g kg^{-1}	12 weeks + 14 days *L. monocytogenes* challenge	N/I	African catfish (*Clarias gariepinus*)	↑ final weight ↑ weight gain ↑ SGR ↑ Feed intake ↑ survival against *L. monocytogenes*	↑ intestine villus length ↑ intestine villus width ↑ intestine absorption area	Improve the fish performance, health, and immune response	Abdel-Tawwab, Adeshina (85)
Clove basil (*Ocimum gratissimum*)	Essential oils	0.5%, 1.0% and 1.5%	55 days + 10 days *S. agalactiae* challenge	1,8-cineole, eugenol and β-selinene	Nile tilapia (*Oreochromis niloticus*)	N/I	↑ Intestine villus height ↑ Intestine goblet cells number ↓ Gill epithelial detachment in the secondary lamellae ↓ Gill congestion at the base of the secondary lamellae	Ameliorate tissue damages, even in situations of infection	Brum, Cardoso (86)
American basil (*Ocimum americanum*)	Essential oils	0.25, 0.5, 1.0 and 2.0 g kg^{-1}	7 weeks	Linalool, eugenol and 1,8-cineole	Red drum (*Sciaenops ocellatus*)	No effect	↑ Stomach lysozyme activity No effect upon the intestinal microbial community	Different supplementation levels do not influence growth performance and intestinal microbial community; however, show effects on immunological responses	Sutili, Velasquez (87)
Savory (*Satureja khuzestanica*)	Powder	1%	45 days	N/I	Common carp (*Cyprinus carpio*)	N/I	↑ Intestinal lactic acid bacteria	Improves intestinal health	Mousavi, Mohammadiazarm (88)
***Allium* sp.**									
Garlic (*Allium sativum*)	Aqueous extract	0.10, 0.15, and 0.20 ml kg^{-1}	80 days	N/I	Guppy (*Poecilia reticulata*)	No effect	↑ Skin mucus lysozyme ↑ Skin mucus alternative complement ↑ Skin mucus total Ig ↑ Skin mucus alkaline phosphatase	Administration of 0.15 mL of garlic extract per kg feed is suggested to obtain optimal skin mucus immunity	Motlag, Safari (89)
Garlic (*Allium sativum*)	Oil	50 µl kg^{-1}	28 days + exposure to silver nanoparticles	N/I	Rohu (*Labeo rohita*)	N/I	↓ Gill oxidative stress enzymes activity ↓ Gill histopathologic lesions	Amelioration of silver nanoparticles-induced oxidative stress and histoprotective effects	Khan, Qureshi (90)
Garlic (*Allium sativum*)	Powder	0.5 g and 1.0 g/100 g	2 months + 2 weeks *S. iniae* challenge	N/I	Nile tilapia (*Oreochromis niloticus*)	↑ Survival against *S. iniae*	↑ Anterior intestine transcriptional levels of interleukin genes (IL-10 and IL-17F) ↑ OTU counts for the phylum of *Proteobacteria* and *Tenericutes*	Could be effective in the prevention of *S. iniae* infection in fish	Foysal, Alam (91)
Garlic (*Allium sativum*)	Powder	5.0%, 10.0% and 20.0%	14 or 28 or 32 days + *C. irritans* challenge	Allicin (1.25 mg/g)	Guppy (*Poecilia reticulata*)	↓↑ Gills and caudal fin *C. irritans* infection	N/I	No clear preventative effect against *C. irritans*	Kim, Fridman (92)

(Continued)

TABLE 1 | Continued

Phytogenic plant origin	Supplemented form	Inclusion dosage(s) tested	Period of administration	Main bioactive components (≤ 3)	Fish species	Performance	Mucosal parameters evaluation	Key benefits summary	Reference
Garlic (*Allium sativum*)	Powder	1.0%, 1.5% and 2.0%	120 days		Rainbow trout (*Oncorhynchus mykiss*)	↑ Weight gain ↑ SGR	↓ Bacterial diversity and richness ↑*Deefgea*, *Mycoplasma*, *Exiguobacterium* and *Clostridium* genera ↓↑ *Aeromonas* genus	Beneficial in terms of promoting growth and inducing changes in the intestinal microbiota in a dose-dependent manner	Büyükdeveci, Balcázar (93)
Garlic (*Allium sativum*)	Powder	5.0, 10.0 and 15.0 g kg^{-1} diet	8 weeks	N/I	Caspian roach (*Rutilus rutilus*)	↑ Weight ↑ Growth rate	↑ Skin mucus antibacterial activity against *S. faecium*, *M. luteus*, *S. marcescens* and *E. coli* ↑ Skin mucus protein level ↑ Skin mucus alkaline phosphatase activity	Beneficially affects the skin mucus immune parameters and growth performance	Ghehdarijani, Hajimoradloo (94)
Garlic (*Allium sativum*)	Lyophilized	2.0%	21 days + cadmium exposure	N/I	Prussian carp (*Carassius gibelio*)	N/I	↓ Gill histopathologic lesions	Shows chelating and antioxidant potential	Nicula, Dumitrescu (95)
Onion (*Allium cepa*)	Powder	1%	45 days	N/I	Common carp (*Cyprinus carpio*)	N/I	↑ Intestinal lactic acid bacteria	Improves intestinal health	Mousavi, Mohammadiazarm (88)
Onion (*Allium cepa*)	Ethanolic extract	0.5%, 1.0%, 1.5% and 2.0%	12 weeks	N/I	African sharptooth catfish (*Clarias gariepinus*)	No effect	↓ Intestine villus length ↑ Intestine villus width ↑ Intestine absorption area ↑ Intestine cryptal depth	Increase the digestive and absorptive capacity of the intestine	Bello, Emikpe (96)
Mongolian Wild Onion (*Allium mongolicum*)	Ethanolic extract	0.04 g kg^{-1}	4 weeks + chromium (Cr) exposure	Flavonoids ≥90% (HPLC)	Grass carp (*Ctenopharyngodon idella*)	N/I	↓ Intestine and gill Cr accumulation ↓ Gill malondialdehyde content ↓ Gill protein carbonyl content ↑ Intestine lysozyme activity ↑ Intestine complement 3 levels ↑ Intestine and gill tight junction proteins gene expression ↑↓ Intestine and gill NF-κB signaling pathway gene expression	Decrease in Cr-accumulation, oxidative stress, immunosuppression and inflammatory response following Cr exposure	Zhao, Yuan (97)
Single bioactive compounds									
Thyme (*Thymus vulgaris*)*	Ethanolic solution	0.15, 0.3, 0.45, 0.6, 0.75 g kg^{-1}	56 days + 14 days *A. veronii* challenge	Thymol (commercial product)	Snakehead fish (*Channa argus*)	↑ Final weight ↑ Weight gain ↑ SGR ↑ Protein efficiency ratio ↓ FCR ↑ Survival *to A. veronii*	↑ Intestine SOD, CAT, GSH-Px activities ↓ Intestine malondialdehyde content ↑ IL-10 and TGF-b gene expression in intestine ↓ HSP70, TNF-a, IL-1b and IL-8 gene expression in intestine	Adequate dietary supplementation can effectively enhance the growth, antioxidant status, immune response and disease resistance	Kong, Li (98)
Thyme (*Thymus vulgaris*)*	N/I	0.1, 0.2 and 0.3 g kg^{-1}	60 days + 8 days *A. hydrophila* challenge	Thymol (commercial product)	Grass carp (*Ctenopharyngodon idella*)	↑ Final weight ↑ Survival against *A. hydrophila*	↑ Gill enzymes of the phosphotransfer network: cytosolic and mitochondrial creatine kinases, adenylate kinase activities and ATP levels in infected fish ↓ Gill ROS levels in infected fish	Favors weight gain and fish longevity. Prevents *A. hydrophila* induced branchial bioenergetics. High concentrations deserve attention because of side-effects	Morselli, Baldissera (99)
Thyme (*Thymus vulgaris*)*	Crystals	0.5 g kg^{-1}	70 days	Thymol (99% purity; commercial product)	Nile tilapia (*Oreochromis niloticus*)	↑ SGR ↑ Protein efficiency ratio	↓ Intestine total aerobic and anaerobic counts	Improve some performance parameters and negatively modulates intestinal microbial communities. Demonstrates a notable synergistic interaction with chitosan nanoparticle with beneficial effects	El-Naby, Al-Sagheer (100)
Thyme (*Thymus vulgaris*)*	N/I	1.0 g kg^{-1}	56 days	Thymol (commercial product)	Rainbow trout (*Oncorhynchus mykiss*)	↓ FCR	↓ Intestine culturable anaerobe bacteria	Modulated intestinal microbial communities disfavoring total anaerobes	Giannenas, Triantafillou (101)
Oregano (*Origanum vulgare*)*	N/I	1.0 g kg^{-1}	56 days	Carvacrol (commercial product)	Rainbow trout (*Oncorhynchus mykiss*)	↓ FCR	↓ Intestine culturable anaerobe bacteria	Modulated intestinal microbial communities disfavoring total anaerobes	Giannenas, Triantafillou (101)
Chinese skullcap (*Scutellaria baicalensis*)*	Powder	0.4, 0.8 and 1.6 g kg^{-1}	60 days + H_2O_2 challenge	Baicalin (80% purity, commercial product)	Nile tilapia (*Oreochromis niloticus*)	↓ FCR	↑ Gill glutathione level ↑ Gill total antioxidant capacity	Improves feed efficiency, enhance antioxidative ability and alleviate oxidative stress	Jia, Du (102)
Garlic (*Allium sativum*)*	Liquid	0.005%, 0.01% and 0.02%	30 days	Allicin (98% purity, commercial product)	Large yellow croaker	↑ Final weight ↑ Final length	↑ Intestine total antioxidant capacity ↑ Intestine antioxidant enzymes (CAT, NO and NOS) activity	Improve the survival and growth of large yellow croaker larvae probably by promoting intestinal development,	Huang, Yao (103)

(Continued)

TABLE 1 | Continued

Phytogenic plant origin	Supplemented form	Inclusion dosage(s) tested	Period of administration	Main bioactive components (≤ 3)	Fish species	Performance	Mucosal parameters evaluation	Key benefits summary	Reference
					(*Larimichthys crocea*)	↑ SGR ↑ survival rate	↓ Intestine transcriptional levels of pro-inflammatory genes	alleviating inflammation and enhancing appetite	
Phytogenics combinations									
Phytogenics combination	Essential oils	200 ppm	70 days + 15 days *N. girellae* challenge	Garlic and Lamiaceae-plants oils (N/I; commercial additive)	Greater amberjack (*Seriola dumerili*)	No effect	↑ Skin mucus lysozyme activity 15 days post *N. girellae* challenge ↑ Piscidin gene expression in skin pre-challenge ↑ Proinflammatory cytokines (*tnf-α* and *il1-β*), AMPs (*hep* and *cath*), immunoglobulin (*IgT*), complement protein (*c3*) T-cells marker (*cd8*) and mucin (*muc-2*) gene expression in skin post-challenge ↓ *Casp3* gene expression in skin post-challenge	Facilitates the immunological response of skin once the parasite is fixed, generating a hostile microenvironment in skin and lowering the parasite load	Fernández-Montero, Torrecillas (16)
Phytogenics combination	Essential oils	0.3 g kg^{-1}	8 weeks + 2 weeks hypoxia challenge (Total 10 weeks)	Cinnamaldehyde, thymol and carvacrol	Nile tilapia (*Oreochromis niloticus*)	↓ Hepatosomatic index	↑ Intestine villi density ↓ Intestine malondialdehyde content	Positive effects of digestion and antioxidative capacity	Ning, Zhang (104)
Phytogenics combination	Microencapsulated essential oils	0.5%	65 days	Garlic essential oil (N/I), carvacrol and thymol (Commercial additive)	Gilthead seabream (*Sparus aurata*)	No effect	↑ Skin mucus inhibitory activity against *V. anguillarum* and *P. anguilliseptica* ↓ Cortisol in skin mucus ↑ Regulation of genes associated to the secretory pathway in skin ↑ Regulation of genes associated to non-specific immune response in skin ↑ Regulation of genes coding for oxidative stress enzymes in skin	Beneficially affects the skin and mucus immune and stress parameters, suggesting the stimulation and recruitment of phagocytic cells and a reduction in the fish allostatic load	Firmino, Fernández-Alacid (14)
Phytogenics combination	Microencapsulated essential oils	0.5%	65 days	Garlic essential oil (N/I), carvacrol and thymol (Commercial additive)	Gilthead seabream (*Sparus aurata*)	No effect	↑ Regulation of genes related to processes of proteolysis and inflammatory modulation, immunity, transport and secretion, response to cyclic compounds, symbiosis, and RNA metabolism in the mid-anterior intestine No effect upon alpha diversity of bacterial communities in the anterior and posterior intestinal tract sections ↓ Spirochaetes phylum in the posterior intestine ↑ *Photobacterium* and *Corynebacterium* genera in the anterior intestine ↓ *Comamonas* in the anterior intestine, and *Paracoccus*, *Prevotella* and *Rothia* genera in the posterior intestine ↓ Bacterial sequences related to carbohydrate and drug metabolisms, and membrane transport ↑ Bacterial sequences related to glutathione and lipid metabolisms, naphthalene degradation and sulphur relay system Evidence of host-microbial co-metabolism	The activation of leukocytes and crosstalk between gut and microbiota are suggested to regulate the inflammatory response induced by the additive	Firmino, Vallejos-Vidal (12)
Phytogenics combination	Microencapsulated essential oils	0.5%	65 days + 39 days *S. chrysophrii*	Garlic essential oil (N/I), carvacrol and thymol (Commercial additive)	Gilthead seabream (*Sparus aurata*)	↓ *S. chrysophrii* total parasitation	↑ Regulation of genes related pro-inflammatory immune response arbitrated by degranulating acidophilic granulocytes, sustained by antioxidant	Promotes gill mucosal immunity and reduces gill ectoparasite incidence	Firmino, Vallejos-Vidal (15)

(Continued)

TABLE 1 | Continued

Phytogenic plant origin	Supplemented form	Inclusion dosage(s) tested	Period of administration	Main bioactive components (≤ 3)	Fish species	Performance	Mucosal parameters evaluation	Key benefits summary	Reference
			challenge (total 104 days)				and anti-inflammatory responses in gills ↑ Carboxylate glycoproteins containing sialic acid in mucous and epithelial gill's cells		
Phytogenics combination	Essential oils	0.02%	9 weeks + 1 week stress and *V. anguillarum* challenge	Garlic and Lamiaceae-plants oils (N/I; commercial additive)	European sea bass (*Dicentrarchus labrax*)	↑ Survival against *Vibrio anguillarum* when stress-challenged	↑ Skin mucus lysozyme activity when stress and bacterial challenge	Attenuate the fish physiological response to stress increasing resistance to *Vibrio anguillarum* infection	Serradell, Torrecillas (105)
Phytogenics combination	Essential oils	0.02%	63 days	Garlic and Lamiaceae-plants oils (N/I; commercial additive)	European sea bass (*Dicentrarchus labrax*)	No effect	↓ Shannon alpha diversity of mucosa-associated microbiota ↑Clostridiales order in intestinal content ↓ Coliforms and Vibrionales allochthonous microbiota	Reduction of orders containing potentially pathogenic species for fish, and enrichment of gut microbiota composition with butyrate producer taxa	Rimoldi, Torrecillas (106)
Phytogenics combination	Essential oils	200 ppm	63 days + 7 days stress and *V. anguillarum* challenge	Garlic and Lamiaceae-plants oils (N/I; commercial additive)	European sea bass (*Dicentrarchus labrax*)	No effect	↓ Posterior intestine fold area covered by goblet cells ↓ Posterior intestine goblet cells area ↑ Intestine mucus coverage post-challenge	Protective effect focused mainly on the preileorectal valve region	Torrecillas, Terova (13)
Phytogenics combination	N/I extract	6.0 g kg ^{1}	30 days + 10 days crowding stress (40 days total)	Saint John's wort (*Hypericum perforatum*, Hypericacea), lemon balm (*Melissa officinalis*, Lamiaceae) and rosemary (*Rosmarinus officinalis*, Lamiaceae) mixed at a ratio 3:2:1	Atlantic salmon (*Salmo salar*)	No effect	↓ Gut lipid peroxidation	Improves the gut antioxidant status	Reyes-Cerpa, Vallejos-Vidal (107)
Phytogenics combination	Powder	1%	45 days	Savory (*Satureja khuzestanica*, Lamiaceae) 0.5% and Onion (*Allium cepa*, Alliaceae) 0.5%	Common carp (*Cyprinus carpio*)	N/I	↑ Intestinal lactic acid bacteria	Improves intestinal health	Mousavi, Mohammadiazarm (88)
Phytogenics combination	Essential oils	0.06, 0.2, 0.4 and 0.8 g kg ^{1}	6 weeks	Thymol and carvacrol (1:1; commercial additive)	Hybrid tilapia (*O. niloticus* ♀ × *O. aureus* ♂)	N/I	↑ Posterior intestine villus height ↑ Posterior intestine goblet cell count per villus ↑ Anterior intestine intraepithelial leucocytes ↓ Distal intestine intraepithelial leucocytes ↑ OTUs, and PD whole tree and Chao1 diversity indexes ↓*Thermi* phylum and *Bacteroides*, *Candidatus Cardinium*, and *Leptospirillum* genera	Affect the immunity primarily through a direct effect on host tissue but also has an indirect effect mediated by microbial changes	Ran, Hu (108)
Phytogenics combination	Essential oils	100 ppm	9 weeks	25% thymol and 25% carvacrol (commercial additive)	Gilthead seabream (*Sparus aurata*)	↓ FGR	↑ Intestine mucosal foldings ↑ Intestine enterocytes ↑ Intestine goblet cells ↓ Expression of genes related to cell differentiation and proliferation, intestinal architecture and permeability, immunosurveillance, such as cytokines, in the intestine	Induce an anti-inflammatory and anti-proliferative transcriptomic profile with probable improvement in the absorptive capacity of the intestine	Perez-Sanchez, Benedito-Palos (109)

SGR, Specific Growth Rate.

FCR, Feed Conversion Ratio.

FGR, Feed Gain Ratio.

OTU, Operational Taxonomic Unit.

**Indicate the putative plant species with high content of the referred bioactive compound. N/I, not identified or not assessed.*

Studies reporting the application of plant extracts or related compounds as bath treatments or evaluating bactericidal or antiparasitic effects in vitro *were excluded from the selection. The table omits systemic immunity-related results, digestive enzymes or other complementary analysis performed within each study. Blends with other components besides the selected group of plants, terpenes or organosulfur compounds were excluded as well.*

(57, 59, 66, 72, 74–80). Besides the assessment of key performance indicators and general immune markers in skin mucus, few studies have tried to explain and characterize the immunomodulatory mechanisms underlying such responses neither which specific compounds might be exerting such effects.

Carvacrol, thymol, p-cymene and γ-terpinene are identified as the predominant bioactive compounds of most of the members of the Lamiaceae family considered in this review, which were mainly found in oregano and thyme. In addition, peppermint, rosemary and basil contain preponderant concentration of other bioactive compounds such menthol, eugenol and 1,8-cineole (**Table 1**). Interestingly, some studies have reported carvacrol and 1,8-cineole presence in the fish skin mucus (64, 82). This phenomenon of bioactive compounds efflux through skin could be responsible for the immunomodulatory and antimicrobial effects observed in the fish skin mucus. However, most of the reviewed studies did not report the phytogenics composition neither the assessment of their translocation through mucus.

Some studies have also reported a protective effect through the reduction in gills' histopathological lesions induced by toxic element exposure or pathogenic challenges (63, 67, 86). Contrarily to the studies describing the effects of phytogenics upon skin mucus secretion and their immunomodulatory potential, their impact upon the GIALT is very scarce, being mostly limited to histological observations. Similarly, studies on the impact of phytogenics upon the intestine are commonly focused on evaluating alterations in morphoanatomical parameters such as an increase in villus length, width and goblet cells count, which are usually associated to improvements in fish growth performance (60, 61, 65, 85, 86). Some studies have also reported the modulation in the number of intestinal lymphocytes (71, 73). Other authors have described a positive impact upon the gastrointestinal activity of humoral immune markers, such lysozyme (87), the activity of antioxidant enzymes and oxidative markers (68, 107) or the down-regulation of the expression of pro-inflammatory genes, such *tnfα* and *tnfβ* (62). Besides, the beneficial impact of phytogenics administration upon the intestine microbiota composition was also suggested (62, 88). Nonetheless, analysis described were constantly incomplete in terms of mucosal immune response evaluation, since limited classical immune or oxidative markers were assessed in each of the above-mentioned studies. Similarly, microbiota studies were often restricted to a particular group of bacteria, such lactic bacteria, failing to properly characterize microbiota functionality and modulation by the experimental diets; thus, resulting in partial and biased conclusions when assessing the regulatory effects of functional feed additives on mucosal tissues.

In addition, some studies reported no effect of the administrated phytogenics upon mucosal parameters (69, 70, 81, 83, 84). Such discrepancies among studies evaluating a particular plant extracts may be due to the diversity of the referred studies in terms of experimental design, fish species selected, plant origin, supplemented form and inclusion level of phytogenetics, among others. This miscellaneous of studies and the lack of protocols allowing appropriate additive and animal testing, highlights the urgent need to standardize the experimental designs and procedures in order to properly evaluate these compounds under *in vivo* conditions and acquire relevant data for their further development and general and safe use.

The effect of some single specific bioactive compounds related to aromatic plants of the Lamiaceae family, such thymol or carvacrol, upon mucosal tissues were also reported (**Table 1**). Although these studies have the advantage to associate a specific mucosal response to the administration of a specific compound, once again it is observed that most of them only reported the analysis of few immune and oxidative markers (98, 99, 102, 104), or a limited microbiological examination (100, 101). In fact, few studies were observed to apply complementary analysis, or achieved to successfully discuss the multifactorial impact exerted by such nutritional strategies upon mucosal tissues (108, 109).

Although the overall data suggest the therapeutic potential of phytogenics derived from Lamiaceae family of plants in aquafeeds, especially of their associated terpene phenolic compounds, unfortunately none of the studies has proposed accurate mechanisms that could be responsible for the broad effects of these metabolites described upon fish mucosal tissues. Despite the lack of reliable information for aquaculture relevant fish species, it is possible that the above-mentioned antimicrobial properties of these compounds, their free radicals' scavenging ability, along with their aptitude to activate TRP channels that modulate inflammatory processes may underlie the immunomodulatory properties and microbiota modulation described in different mucosal tissues.

Effect of Dietary Organosulfur Compounds Upon Fish Mucosal Immunity

The main constituents of extracts and essential oils from *Allium* sp., such as garlic (*Allium sativum*, Alliaceae) and onion (*Allium cepa*, Alliaceae), are sulfur-containing compounds. This group of bioactive substances comprises alliin, allicin and its derived bioactive compounds like ajoene, diallyl trisulfide (DATS), diallyl disulfide (DADS), diallyl sulfide (DAS) and allyl methyl disulfide, commonly termed as organosulfur compounds. These organosulfur compounds are the responsible for the recognized antioxidative, antimicrobial, antifungal and antiparasitic properties of garlic (127). Allicin (S-allyl-2-propenyl thiosulfinate) is usually the main biologically active component of garlic and related species; however, it is highly unstable under physiological conditions; thus, quickly being transformed into its organosulfur derivates, which also exhibit therapeutic properties (128–130).

Organosulfur compounds have been particularly studied for their antiparasitic activity. Among them, ajoene was described to interfere with parasite and host cell membrane protein and lipid trafficking, with irreversible detrimental consequences for the parasite (131). This is of special relevance since teleost mucosal tissues are known to have a high constitutive expression of Th2 markers that indicate a skewed immune response targeted against parasites (132). Regarding their bactericidal properties, the organosulfur compounds can penetrate the bacterial cell membranes, cause changes in the structure of thiol (-SH)

containing enzymes and proteins, and lower the expression of important genes involved in the QS in bacteria, inhibiting the growth of both Gram-positive and Gram-negative bacteria (133). The higher the number of sulfur atoms present in the compounds, the more is its bactericidal activity (134). Therefore, the administration of organosulfur-containing phytogenics may induce important changes in the fish mucosal-associated microbiota with potential effects upon the mucosal immunity.

The detoxification and chemoprotective benefits from various organosulfur compounds have been associated to their ability to scavenge free radicals and selectively enhance or suppress the levels genes or proteins of several antioxidant enzymes, such as cytochrome P450 enzymes or glutathione S-transferase (GST) (135), exerting a direct effect upon immune cells (136). In this line, their anti-inflammatory activity upon immune and intestinal epithelial cells was associated to the inhibition of ROS production and the modulation of the NF-*k*B and MAPK signaling pathways (137, 138). Some organosulfur compounds, such allyl sulphides, were also observed to increase the levels of anti-inflammatory H_2S in intestinal epithelial cells, promoting mucosal integrity, tissue repair and stimuli perception (139). In accordance, organosulfur compounds, such as DADS, are also donors of H_2S, whose positive effects upon the intestinal health could be also produced through the modulation of the enteric microbiota (140). In addition, organosulfur compounds were observed to promote mucin expression in human airway epithelial cells, being suggested to improve the mucosal epithelial barrier function (141, 142).

Conversely, these organosulfur compounds have been also reported to stimulate inflammatory immune responses, promoting the release of pro-inflammatory cytokines, enhancing the proliferation of lymphocytes, macrophage phagocytosis and modulating the infiltration of immune cells (143). For instance, it was demonstrated that some allyl-containing organosulfur compounds directly activate Ca^{2+} flux in neutrophils augmenting their phagocytic function and consequent ROS production. In parallel, other compounds are able to inhibit spontaneous ROS production by neutrophils (144). This apparent antagonistic effect evidences the pleiotropic protective effects of garlic extracts and essential oils, being simultaneously capable of inducing immune responses and anti-inflammatory counteractions. Moreover, as previously suggested for the mode of action of terpene phenolic compounds, organosulfur compounds are also able to activate TRP channels, TRPA1 and TRPV1 channels in particular (145–147), suggesting the Ca^{2+} induced cellular immune activation (144).

Garlic has been for long studied and recognized for its benefits as growth- and flesh quality-promoting effects in cultured fish, as well as for its antibacterial and antiparasitic properties (51). However, there is scarce information regarding the activity of garlic-derived organosulfur bioactive compounds upon fish mucosal tissues. The synthesis of the results from several studies reporting the health promoting properties of phytogenics derived from *Allium* sp. are shown in **Table 1**. Similar to studies testing phytogenics from Lamiaceae family origin, the evaluation of the supplementation of phytogenics derived from *Allium* sp., also focuses on few immune markers in skin mucus (89, 94), histopathological observations or some inflammatory and oxidative markers in gills (90, 97) and intestine (91, 95–97, 103), or incomplete microbiological examination that lacks in-depth the functional interpretation of their mode of action at cellular level (88, 91, 93).

Overall, studies reporting the effect of the administration of phytogenics derived from *Allium* sp. upon fish mucosal tissue suggest the health-promoting potential of the organosulfur compounds that characterize this group of plants. However, there is currently no robust studies under a pharmacological point of view that intent to demystify the accurate mechanisms responsible for the effects described on fish mucosal tissues, whose lack of reliable information critically restrains their application as potential functional feed additives in aquafeeds. Similar to the terpene phenolic compounds, the organosulfur compounds have also recognized antipathogenic and antioxidant properties, in addition to their common ability to activate TRP channels that modulate inflammatory processes. Since in higher vertebrates dermal emission of organosulfur compounds were demonstrated after garlic ingestion (148). In this sense, the efflux of organosulfur compounds through the integument could be also playing a critical role in the recurrently reported effects of dietary garlic and other aromatic plants in fish mucus, as previously referred for carvacrol and 1,8-cineole.

Effect of Combinations of Phytogenics Derived From Lamiaceae and *Allium* sp. Upon Fish Mucosal Immunity

Some studies have reported the beneficial effects of the combination of Lamiaceae and *Allium* sp. phytogenics upon fish mucosal tissues (**Table 1**). For instance, in European seabass (*Dicentrarchus labrax*) a combination of garlic and essential oils from plants of the Lamiaceae family promoted skin mucus lysozyme activity and fish survival against *V. anguillarum* when exposed to a confinement stress (105). In greater amberjack (*Seriola dumerili*), the same additive induced an up-regulation of a set of immune related genes in the skin in response to a monogenean parasite *Neobenedenia girellae* infection (16). In accordance, a blend of garlic essential oils, carvacrol and thymol was also reported to positively impact both gilthead seabream (*Sparus aurata*) skin mucus in terms of bacterial inhibition capacity against fish pathogens and decrease of stress markers, whereas the transcriptional analysis suggested the stimulation of the secretory pathway possibly associated to humoral immune molecules secretion into mucus and activation of phagocytic cells (14). The same blend was reported to regulate the transcription of genes related to immune response in gills, which was mediated by granulocytes, as well as sustaining both anti-inflammatory and antioxidative responses. In addition, the above-mentioned study revealed that the tested phytogenic compounds promoted the presence of sialic-acids containing glycoproteins in both epithelial and mucous cells, which globally resulted in a decrease in the intensity of gills' infestation by the monogenean ectoparasite *Sparicotyle chrysophrii* in gilthead seabream (15). Both, referred

phytogenics combinations were observed to positively affect the gut health status of those fish species by improving the protective intestine mucus coverage post-challenge (13), regulating the intestine immune transcription (12) and modulating their intestinal microbiota (12, 106).

According to the above-described studies and the acknowledged properties of these phytogenics' bioactive compounds, we suggest that the mechanisms of cell activation that may be responsible for the mucosal immune-related responses are mediated by the activation of TRP cation channels in both immune and epithelial cells of mucosal tissues. The bioactive compounds may activate TRP channels leading to intracellular Ca^{2+} increase and the activation of the TAK1/MAPK/NF-*k*B signaling pathways, modulating the expression of pro-and anti-inflammatory cytokines, and antioxidative enzymes such as cytochrome P450. In parallel, stimulation by pathogen-associated molecular patterns (PAMPs), who might be also modulated by the antimicrobial properties of these compounds, may facilitate the activation of TLR and TRP signaling pathways; thus, amplifying the mucosal immune responses. Moreover, the bioactive compounds are also suggested to passively diffuse across the cell membrane, scavenging ROS that contribute to the inflammatory pathways, and interacting with TRP channels of the endoplasmic reticulum, potentially stimulating the secretory pathway. The above-described mode of action of phytogenics derived from Lamiaceae family and *Allium* sp. at the level of the main mucosal lymphoid tissues in fish is depicted in **Figure 1**.

In addition, a summarized representation of the potential mucosal immune responses induced by the dietary administration of terpene and/or organosulfur based phytogenics and their effects against fish pathogenic organisms is suggested in **Figure 2**. In this representation, the holistic perspective of the compounds' effects upon the most studied mucosal-associated lymphoid tissues in fish so far – gill, gut, and skin – as targets for oral immunostimulation is highlighted through the stimulation of both humoral and cellular immunity, mucosal secretion, microbiota modulation and other potential physiological and metabolic responses.

FUTURE PERSPECTIVES

Feeding the projected world population by 2050 in a sustainable way is a great challenge, in which aquaculture is predicted to supply the majority of aquatic dietary protein. For that, the implementation of novel policies and production system approaches targeting

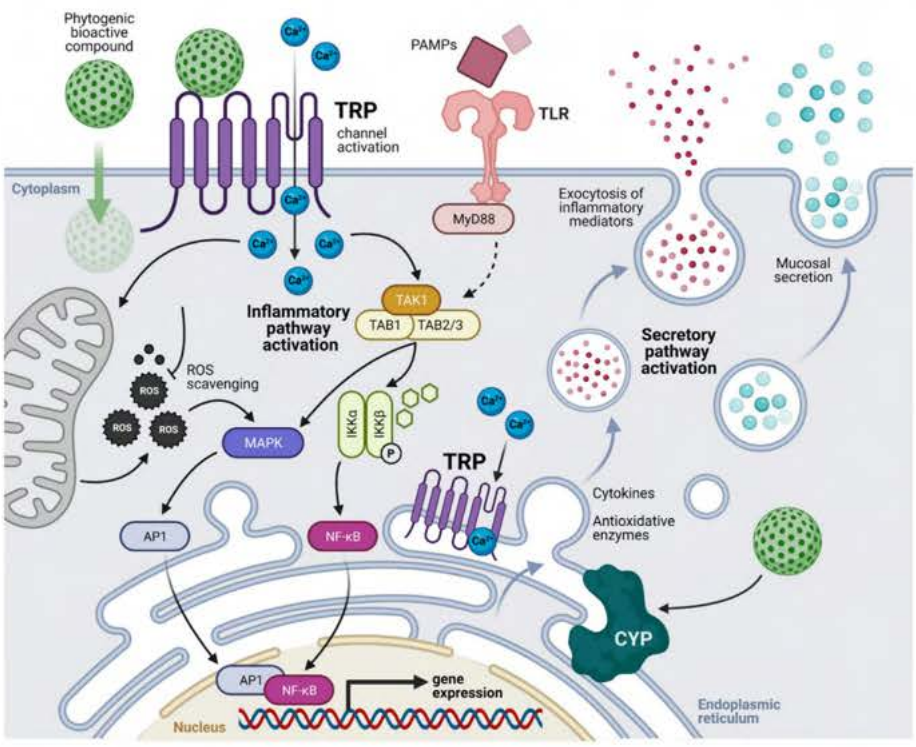

FIGURE 1 | Suggested mechanisms of cell activation by the transient receptor potential (TRP) cation channels mediated by phytogenics' bioactive compounds in mucosal-associated lymphoid tissues (MALTs). Bioactive compounds activate TRP channels leading to intracellular Ca^{2+} increase and non-canonical activation of the TAK complex. In parallel, stimulation by pathogen-associated molecular patterns (PAMPs) may facilitate the activation of TLR and TRP signaling pathways. Modified from Galindo-Villegas, et al. (125). TLR, toll-like receptors; MyD88, myeloid differentiation primary response 88; TAK, transforming growth factor beta (TGFβ) activated kinase; TAB, TGFβ activated kinase binding protein; ROS, reactive oxygen species; NF-*k*B, nuclear factor kappa-B; IKK, inhibitor of NF-*k*B kinase; MAPK, mitogen-activated protein kinase; AP1, activator protein 1; CYP, cytochromes P450; P, phosphorylation.

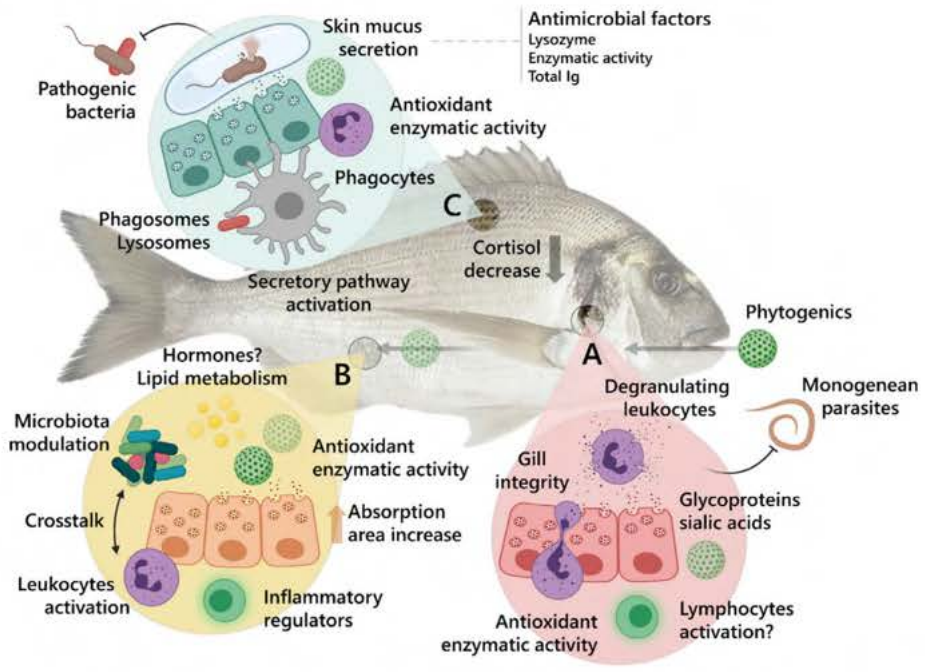

FIGURE 2 | Summary of the proposed mode of response induced by phytogenic bioactive compounds in fish mucosal tissues. The effects against representative types of common pathogens on the most studied mucosal-associated lymphoid tissues in fish so far are represented in the figure. **(A)** Gills. **(B)** Gut. **(C)** Skin. Gilthead seabream (*S. aurata*) was used in the figure as a representative aquaculture-relevant fish.

effective health management and animal welfare are mandatory (1). Moreover, indiscriminate prophylactic use of antibiotics associated to intensive aquaculture practices can still be observed among some of the major aquaculture producing countries, as it has been recently reviewed (149, 150). However, in 2022, several countries, including the EU will prohibit all forms of routine antibiotic use in farming, including preventative group treatments which highlights the necessity for the development of more sustainable alternative treatments (151). Under this context, the market for sustainable products and feed additives is increasingly growing and the number of studies on the use of a wide variety phytogenics as sustainable tools to be implemented in aquaculture production has increased dramatically in the last decade (5). The global market of phytogenic feed additives including major tier I and II suppliers was estimated on 753M USD in 2020 and it is projected to reach 1,098M USD by 2025 (152).

Although limitations in testing and reproducing studies using dietary immunostimulants have been pointed out since long ago (153–156), the current knowledge on the pathways and mechanisms followed by these compounds at the cellular level is still neglected. A large selection of experimental designs, fish species, phytogenics tested and diet composition fails in the association of selected bioactive compounds to specific effects. Moreover, it is important to consider the difficulties to carry out comparison among the available studies because of the different assays, testing methods, different extraction procedures of plant essential oils or extracts, and the intrinsic variation in chemical phytoconstituents in plants due to different agroclimatic conditions, harvesting season and plant phenotype. This essential oils or extracts consist of a variable mixture of different bioactive molecules that area generally not characterized, and are administrated through variable periods of time, dietary doses and forms in different fish species that are generally randomly selected. In addition, a considerable number of these studies provide little or partial information regarding the effect of a given immunostimulant, since their definitive efficiency assessment rely on the evaluation of basic biochemical parameters that are to some extent obsolete if compared against the actual state-of-the-art. Based on the former idea, diverse omics tools available play a fundamental role for proper understanding and characterization of their mode of action in mucosal tissues at cellular level. Together, these factors question the reproducibility and safety of a large number of studies available and limits the use of several of those proposed substances in commercial functional feeds.

In this regard, we propose that the study of purified bioactive compounds may represent a viable solution to circumvent variability, and the biological mode of action of isolated compounds should be primarily assessed *in vitro* under variated settings, before moving forward to *in vivo* trials. However, it is important to consider that the biological activities of essential oils or extracts cannot be attributed to a single compound or to a unique specific mechanism, since their multi-component properties exert greater biological activity when compared to the major components alone, whose function is probably regulated by the synergy with limiting compounds (8). In this light, essential oils and extracts from different plants have been explored for their potential as resistance modifying agents (157).

While their chemical complexity may represent a clear advantage in terms of reducing the risk of inducing bacterial adaptation and resistance to single compounds, or even promoting a wider antibacterial activity, the use of blends of phytogenics makes difficult to proper characterize their mode of action. Therefore, when developing such additives, the formulation of combined purified compounds through the correct and soundly *in vitro* functional characterization to obtain potential synergies is recommended. Moreover, long-term studies assessing whether the bioactive compounds, single or combined, induce bacterial tolerance, transmissible adapted resistance or any other change on a large scale should be implemented and the effects on both beneficial and pathogenic bacteria determined through *in vitro*, *in silico* and finally *in vivo* approaches (104, 158).

While several phytogenics have been proved remarkably efficient in promoting mucosal fitness (9), little efforts have been made to elucidate the underlying pharmacokinetics and immunostimulatory mechanisms of tested compounds upon the MALTs, with few *in vitro* studies published to date. It should also be highlighted that occasionally *in vitro* studies do not accurately translate into predictable responses *in vivo* (84); thus, both *in vitro* and *in vivo* studies should be performed whenever possible. This lack of complementary information supports the demand for additional profound research on the fate and length in which particular phytogenic compounds act, which is crucial for further developing functional additives and their application in an industrial context. Although the specific mechanisms behind the observed fish mucosal physiological responses are still poorly described, it is possible that cellular pathways involving the activation of TRP receptors by the bioactive compounds might be responsible for the reported mucosal immune responses. Besides, this response might be potentiated by the PAMP-induced activation of the TLR cell-signaling cascade, as synthetized in **Figure 1**, which would explain the fish improved ability to cope with pathogenic challenges. Thus, it is advisable in nutritional dose-response evaluating phytogenics to evaluate changes in expression in TRP receptors as well as gene markers of the TAK1/MAPK/NF-*k*B signaling pathways in order to provide insight into their mode of action at mucosal level.

Another limitation that should be taken into account when testing phytogenics is that most plant-derived bioactive compounds are either volatile and/or susceptible to rapid degradation in the stomach where acid digestion takes place, with consequent low availability at the intestinal level or uncontrolled changes in the dose of administration. Hence, to overcome this limitation and minimize potential losses, controlled releasing techniques, such as encapsulation or other coating technologies, can be used to improve the proper delivery of phytogenics. This technology allows a prolonged absorption and local availability of the bioactive compounds along the gastrointestinal tract, ultimately increasing their beneficial impact upon the host (159, 160). Moreover, encapsulation protects phytogenics from environmental degradation, such as from light, temperature and/or pH variations, and eventually playing an important role in their palatability, masking the potential pungency associated to some compounds that otherwise can affect feed intake (159). It is important to highlight that most of the studies considered in this review did not take into consideration those aspects, administrating phytogenics as powder forms, hydroethanolic extracts or dissolved solutions without proper assessment of their potential biodegradation during feed storage or along the gastrointestinal tract. The overall limitations identified in most of the currently available studies assessing fish immunity leads to the stigmatization of phytogenics application, in which compounds with high pharmacological value are labeled under the "medicinal plants" or "herbalism" pseudoscience stigma, with disbelieving scientific evidence. This represents a major restriction for the development of effective phytogenics at commercial scale.

CONCLUSIONS

Overall, it is fundamental that the efforts made in the research for sustainable prophylactic tools to boost host's immune condition, stress resistance and pathogenesis prevention will culminate on reliable administration strategies for the aquaculture sector. Among the most studied group of natural bioactive compounds, both terpenes and organosulfur compounds have been suggested to display antimicrobial, antioxidant, anti-inflammatory and immunomodulating activities, with the potential of improving fish mucosal barrier function and integrity. Although they comprise a promising group of phytogenics for aquafeeds, an urgent update in the academical approach and experimental methodologies are needed to elucidate their pharmacokinetics and mode of action in depth. Therefore, in the present review we propose important molecular signaling pathways and hypothesize their involvement on the dietary immunomodulation in fish by the selected phytogenics.

AUTHOR CONTRIBUTIONS

JF and JG-V conceived the study. JF wrote the draft. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: JF is employed by TECNOVIT-FARMFAES S.L.

DISCUSSION

DISCUSSION

Responding to ever-growing aquaculture challenges: functional feeds as a sustainable strategy for preventing disease outbreaks

In the last 20 years, consumers from low- to high-income countries have benefited from year-round availability and access to aquatic foods due to continuous aquaculture growth [1]. To supply the increasing fish and seafood demand derived from a steadily demographic growth and changes in human seafood consumption patterns, aquaculture will continue to be the driving force behind the growth in global fish production, extending a decades-old trend. World aquaculture production is expected to expand from 179 million tonnes in 2018 to 204 million tonnes in 2030 (15% over 2018) [2]. To provide social and environmental benefits, sustainable aquaculture production practices are mandatory. However, despite the impressive gains mentioned, the aquaculture rapid expansion increases its own vulnerability to many serious challenges that hamper the sector's development. In some cases, undermining its ability to achieve those sustainable necessary outcomes and affecting negatively consumers' opinion and the sector credibility. In fact, the average annual growth rate of aquaculture is projected to slow down from 4.6% in 2007–2018 to 2.3% in 2019–2030. A number of factors are expected to contribute to this slowdown, including the broader adoption and enforcement of environmental regulations, the decrease in the availability of water and suitable production locations, the decrease in productivity gains, the increasing outbreaks of aquatic animal diseases related to intensive production practices, and all the constrains associated to climate change [2].

The intensified production systems and climate change facilitate the occurrence of disease outbreaks due to the favoring of stressed and immuno-compromised animals, and the evolution and spread of more and more virulent pathogens. This qualifies aquatic animal diseases as one of the major limiting factors for aquaculture

development [3]. Despite the efforts deployed in improving disease surveillance and management, a global trend in aquaculture is that previously unreported pathogens that cause new and unknown diseases will emerge and spread rapidly, causing major production losses approximately every three to five years [2]. In order to prevent and mitigate such economic losses, indiscriminate prophylactic use of antibiotics and chemicals associated to intensive aquaculture practices can still be observed among some of the major producing countries [4; 5]. However, the recurrent use of such therapeutics have serious side-effects on the aquaculture system, not only by immunosuppressing animals, but also due to the selection and emergence of more virulent strains and antibiotic-resistant bacteria [3]. In the European Union (Regulation 1831/2003/EC), the antibiotic resistance threats and the ban on antibiotics as growth promoters in animal feeds have being motivating the animal production industry to adopt antibiotic-free productions [3; 6] and the development of more sustainable alternative treatments [7]. With the objective of preventing disease outbreaks and to reduce the use of chemotherapeutic drugs in aquaculture, alternative/complementary strategies have been proposed, such as vaccination and the administration of functional feed supplements [8; 9], among others [10].

Although vaccination has proven to be an excellent prevention tool in some aquaculture sectors, the vaccination is a pathogen-specific technique that requires a clear disease diagnosis, presenting limited efficiency against multi-agent infections. Besides, the time and costs associated to vaccine development may limit its availability and application towards a wide repertoire of pathogenic organisms, resulting in the present-day inexistence of effective vaccines against several economically relevant diseases, such as for viral and parasitic infections [9]. On the other hand, since disease is intimately related to the fish basal physiological and immunological status, the use of functional feeds that provide health benefits beyond their nutritional value has received substantial attention over the last decade [7; 11]. In this scenario, among different functional feed additives that may be used in aquaculture –the reader is encouraged to consult Encarnação [12] or the

Introduction section of this thesis for a detailed description of them–, phytogenics are defined as environmentally friendly plant-derived natural compounds used as functional feed additives that show positive effects on animal growth and health. Phytogenics often comprise aromatic plants extracts and essential oils characterized by its richness in biologically active compounds [13; 14]. In farmed fish, a wide spectrum of phytogenics have been increasingly studied mainly due to their growth-promoting, antimicrobial, immunostimulant, antioxidant, anti-inflammatory and sedative properties [15],. For this wide repertoire of action, phytogenics represent a promising effective and sustainable prophylactic tool to be implemented in health management in front of bacterial and parasitic infections [8].

The mucosal tissues of farmed fish are a target for immunostimulatory dietary manipulations as they are one of the main portals of pathogens' entry into the organism, and are characterized by an intricate immune system associated to lymphoid tissue [16; 17]. In the context of this thesis, key performance indicators, and the transcriptional immune response of three selected mucosal tissues –gill, intestine and skin– were analyzed in one of the most important marine fish species farmed in the Mediterranean, the gilthead seabream (*Sparus aurata*), fed an additive composed by microencapsulated garlic essential oil, carvacrol and thymol (Chapters I, II and III; [18; 19; 20]). These phytogenics are obtained from some of the most studied group of plants, the Lamiaceae family and *Allium* sp. In fact, they have been particularly tested and their effectiveness against parasitic and bacterial infections have been extensively demonstrated both *in vitro* and *in vivo* [21; 22; 23]. The synthetized and critical review of the results from several studies reporting the beneficial effects of the dietary inclusion of phytogenics derived from the Lamiaceae family and *Allium* sp., upon several fish species growth and feeding performance, and mucosal tissues response may be found in Chapter IV (see Table 1 from Chapter IV).

Effects of garlic, carvacrol and thymol on fish key performance

Growth has been traditionally considered as one of the main endpoints and key performance indicators when evaluating different feed formulations and additives' inclusion. Carvacrol, thymol and garlic have been often proposed as growth promoters for several fish species [22; 24; 25; 26; 27; 28; 29; 30; 31; 32; 33; 34; 35; 36; 37]. Most studies reporting benefits upon somatic growth in fish fed these phytogenics, also described a significant decrease in the feed conversion rates (FCR) [33; 34; 38; 39; 40; 41; 42]. Conversely, other studies have reported no effects upon growth nor feeding efficiency for similar fish species [43; 44; 45; 46; 47; 48; 49]. In our study, the dietary inclusion of carvacrol, thymol and garlic essential oil did not affect gilthead seabream growth performance (Chapter I [20]). There are innumerous constrains related to the reproducibility of nutritional trials that may explain such discrepancies among studies [50], such as i) the variable and untrustworthy origin of phytogenics (i.e. raw materials obtained from local markets or tailor-made by authors), ii) the utilization of different supplementation forms (i.e., powder, essential oil, ethanolic extracts, etc.) and inclusion doses, iii) the different periods of administration and diet formulations, among others. The instability of free bioactive compounds, such as the particular case of the garlic organosulfurs, may also lead to contradictory results if nor administrated properly [51]. This highlights the benefits of encapsulating this type of phytogenics, since this process prevents the degradation of bioactive compounds due to inconvenient interactions with the host metabolism and environment, allowing their proper delivery through the gastrointestinal tract [52]. In addition, the utilization of natural additives of unknown (and highly variable) composition represents an important constrain to the reproducibility and robustness of the phytogenics-testing studies, usually failing under industrial and commercial scales. Further aspects of standardization and reproducibility in feed additive development and testing for aquaculture purposes are discussed in Chapter IV.

Nevertheless, it is important to consider that the basal diet in our study was formulated to meet the nutritional requirements of gilthead seabream under

summer conditions (46% crude protein, 18% crude lipids; 30% fishmeal) and, accordingly, specific growth rate (SGR) values of gilthead seabream fed both control and the phytogenics-supplemented diet compared favorably to those reported by Mongile et al. [53], as described in Chapter I [20]. Therefore, the absence of an improvement in growth parameters under our experimental conditions may be related to the absence of a dietary challenge, such as the inclusion of the additive in a low fishmeal diet, which could possibly reveal the growth-promoting potential of the tested phytogenics or even additional health benefits. Regardless of these factors, the phytogenics tested in this thesis were not chosen for their potential positive effect on somatic growth or feeding efficiency, rather than for their antiparasitic and antibacterial properties [54]. In fact, it is commonly acknowledged that the occurrence of disease can negatively affect fish productive parameters and welfare.

One of the main objectives of this thesis was to evaluate the anthelmintic properties of the referred additive against the gill ectoparasite *Sparicotyle chrysophrii*, one of the main pathogenic agents affecting gilthead seabream farming in the Mediterranean sea [55]. Garlic-based treatments have been demonstrated to be particularly effective against monogeneans and other parasites [56; 57; 58; 59; 60; 61]. Several dietary and bath treatments with different essential oils containing carvacrol and/or thymol have been proved to be effective antiparasitic treatments as well [62; 63; 64; 65; 66]. Nonetheless, there are few studies that accurately describe the antiparasitic effect of dietary phytochemicals against monogeneans in fish species [49]; thus, the pathways and mechanisms of their action are not clear yet [67]. Moreover, most of the existing studies are focused in the effects of the above-mentioned phytogenics on intestinal health [25; 68; 69; 70; 71], or in their use in balneation treatments against parasitic organisms [56; 62; 63; 64; 72]. As far as we know, the study reported in Chapter I [20] was the first describing the gill's response in gilthead seabream to the administration of a blend of carvacrol, thymol and garlic essential oil as a feed additive, as well as the potential mechanisms underlying its antiparasitic properties.

In brief, gilthead seabream fed the tested phytogenics-supplemented diet and challenged with *S. chrysophrii* showed a reduction of 78% of total parasite load when compared with the control group, with a decrease in the prevalence of most of the developmental stages of the parasite (Chapter I [20]). The transcriptomic data from gills at the end of the nutritional trial provided the baseline knowledge for deciphering the antiparasitic role of the tested additive, since the transcriptional analysis of the gills showed a predominant up-regulation of genes related to an immune response arbitrated by degranulating acidophilic granulocytes, sustained by antioxidant and anti-inflammatory responses. For several aquaculture relevant species, some studies have reported an increase of blood neutrophil number through the dietary supplementation of garlic or its bioactive compounds, carvacrol and/or thymol [29; 73], or after therapeutic balneation with essential oils reported to be an effective treatment against monogeneans [62; 74; 75]. Furthermore, the histochemical study of the gills of fish fed the phytogenics-supplemented diet also showed an increase of carboxylate glycoproteins containing sialic acid in epithelial cells and hypertrophied mucous cells, suggesting a mucosal defense mechanism through the modulation of mucin secretions promoted by the additive (Chapter I [20]). Other studies in gills have reported a protective effect of these phytogenics or related ones through the reduction in histopathological lesions induced by toxicants or pathogenic challenges, usually associated to an improvement of the gills' antioxidant capacity [76; 77; 78; 79; 80; 81; 82]. Therefore, the stimulation of immune cells in gills, the improvement of their antioxidant capacity and the enhancement of mucosal secretion promoted by the tested feed additive are possibly the main actors responsible for the antiparasitic effect observed when dealing with this kind of phytogenics.

Besides their above-mentioned potential antiparasitic properties, some studies have reported the advantageous outcomes of the dietary administration of garlic, carvacrol, and/or thymol in the gut health of aquatic species [25; 83]. These beneficial effects have also been associated to the modulation of fish microbiota composition [84], as well as to a significant improvement in fish resistance to

intestinal infections [71]. Studies on the effect of those phytogenics upon the intestine have frequently described alterations in morphometric parameters, such as an increase in villus length, width and goblet cells count, usually associated to improvements in fish somatic growth [35; 44; 78; 85; 86; 87; 88]. In addition, some studies have reported the modulation in the number of intestinal lymphocytes in fish species, including gilthead seabream [47; 87; 89]. This would be in accordance with our results described in Chapter II [19], where a transcriptional response involving leukocytes activation (similar to the observed in gills, Chapter I [20]) was also obtained in the mid-anterior intestine of gilthead seabream fed the tested phytogenics-supplemented diet. In agreement with our intestinal transcriptional profile described in Chapter II [19], other authors have also described a positive impact upon the activity and gene expression of immune markers [36; 39; 82; 90; 91; 92] or antioxidant enzymes and other oxidative markers in the intestine of fish [36; 45; 88; 93].

Although commonly not considered as a key performance indicator, the gut microbiota and its modulation through nutritional strategies can significantly influence fish performance. Due to their antibacterial properties, phytogenics can modulate the fish microbiota composition and their metabolites, which, in turn, may interact with the host immune system, potentially improving growth and feed efficiency, or even protecting fish gut from inflammation and pathogenesis [94; 95]. In fact, numerous studies have reported beneficial modulations of the fish intestinal microbiota composition by the dietary supplementation of garlic, carvacrol, and/or thymol [25; 40; 87; 92; 96; 97; 98; 99]. In particular, an additive composed by phytogenics similar to the one evaluated in the present thesis was observed to positively affect the gut health status of European sea bass (*Dicentrarchus labrax*) by modulating their intestinal microbiota [100] and improving the protective intestine mucus coverage after an intestinal *Vibrio anguillarum* infection challenge combined with crowding stress [101]. While in our study gut microbiota alpha diversity indexes were not affected by the tested additive, which was probably due to the heterogeneity of analyzed samples, the phytogenics-supplemented diet promoted

subtle, but significant alterations in microbiota composition. Those variations in microbiota composition were also suggested to participate in the modulation of the intestine transcriptional immune profile through the host-microbial co-metabolism, as further discussed in more detail in Chapter II [19].

Garlic, carvacrol and/or thymol related phytogenics were also demonstrated to significantly improve fish survival to pathogenic challenges after their dietary administration [33; 34; 36; 38; 39; 79; 92; 102; 103]. Several of these studies have also reported an improvement in the fish skin mucus immune markers and/or an increased skin mucus antibacterial capacity against a fish bacterial pathogen in those fish fed the phytogenics [33; 34; 37; 38; 48; 103; 104; 105]. For instance, in European seabass (*D. labrax*) a combination of garlic and essential oils from Lamiaceae plants promoted skin mucus lysozyme activity and fish survival against *V. anguillarum* when exposed to a confinement stress [103]. The same additive also increased the skin mucus lysozyme activity and the gene expression of several mucosal immune markers in the skin of greater amberjack (*Seriola dumerili*) challenged with the monogenean ectoparasite (*Neobenedenia girellae*) [106]. The referred studies are in accordance with the results presented in this thesis, where the analysis of the skin transcriptional profile of fish fed the phytogenic-supplemented diet revealed a regulation of genes recognized by their involvement in non-specific immune responses and the stimulation and recruitment of phagocytic cells (Chapter III [18]). In addition, the promotion of the secretion of a variety of non-specific immune molecules into the skin mucus was proposed to be responsible for the *in vitro* decreased growth capacity of pathogenic bacteria, such as *V. anguillarum* and *P. anguilliseptica*, observed in the mucus of fish fed the phytogenic-supplemented diet, as described in Chapter III [18]. Overall, garlic, carvacrol and/or thymol are suggested to promote mucosal immune responses that reduce the settlement of pathogenic bacteria in the skin surface through the improvement of the mucus biostatic or biocidal activity, therefore decreasing its susceptibility to infection.

In addition, Mizuno et al. [107] associated the antiparasitic effect observed in the skin of chum salmon (*Oncorhynchus keta*) fed a diet supplemented with oregano to the carvacrol content detected in those fish skin mucus. Other studies have also reported a similar exudation of bioactive compounds into fish skin mucus after phytogenic supplementation [108]. This phenomenon of bioactive compounds efflux from the site of absorption in the intestinal tract, through the skin and into the mucus appears to be responsible for the immunomodulatory and antimicrobial effects observed in the fish skin mucus. However, if the efflux of bioactive compounds is occurring, it is possible that it could be affecting the organoleptic properties of the fish meat, and consequently its quality.

In order to assess whether the additive tested in this thesis may negatively impact fish meat quality, a second nutritional trial with commercial-sized gilthead seabream (BW_i = 312.9 ± 0.9 g; BW_f = 471.1 ± 8.9 g) was performed followed by a sensory evaluation of the fillet. Fish were fed the phytogenics-supplemented diet and a control diet for 2 months, and then several periods of additive suppression from the diet were established (7, 14, 21 and 28 days of additive feeding suppression). For each sampling point, fillet samples from both fish sides were vacuum packed and stored at –18°C until their analysis by a trained tasting panel from the Food Science and Technology Research Group of the University of Murcia (Spain). The sensory analysis was carried out using a quantitative descriptive test, a technique that allows the identification and quantification of the sensory characteristics or attributes of a product, such as smell, flesh color, shine, flavor, juiciness, firmness and chewiness. The fillet, steamed for 3 min until reaching an internal temperature of 72°C, from gilthead seabream fed the additive-supplemented diet presented the same sensory attributes as those from fish fed the control diet, whatever the period of additive suppression considered (7, 14, 21 and 28 days). Since the tested additive did not modify the sensory characteristics of the cooked seabream fillet, its use in fish feeding was considered not to represent a risk for the organoleptic quality of the final product (Mª. D. Garrido and J. Firmino, unpublished data). Furthermore, the proximate composition of the fillet revealed

that the inclusion of the additive did not significantly affect the protein (control = 22.0 ± 0.2 %w/w; additive = 21.1 ± 0.5 %w/w) and lipid (control = 1.4 ± 0.1 %w/w; additive = 1.4 ± 0.1 %w/w) levels. Similarly, no significant differences were observed for both fillet ash (control = 5.2 ± 0.3 %w/w; additive = 4.8 ± 0.1 %w/w) nor humidity content (control = 71.9 ± 0.7 %w/w; additive = 72.1 ± 0.4 %w/w) (J. Firmino, unpublished data).

Bioactive compounds from plant origin, such the ones administrated as phytogenics, are also reported having sedative properties [103; 109; 110; 111; 112]. The assessment of fish allostatic load is often based on the measurement of metabolites' levels associated with stress response in the blood plasma, namely cortisol, glucose and lactate [113]. Several studies have reported a significant decrease in the plasma levels of stress-related markers in fish fed garlic, carvacrol and/or thymol supplemented diets [102; 107; 110; 111]. Since a positive correlation between cortisol levels on fish plasma and skin mucus was demonstrated [114] including for gilthead seabream [115], the decreased levels of cortisol in the skin mucus of fish fed the phytogenics-supplemented diet observed in our study indicated a reduction in the fish allostatic load (Chapter III [18]). This improvement in fish stress indicators may result from i) the referred sedative properties of the phytogenics used, ii) the promotion of the fish immunity and/or iii) the potential decrease in skin pathogenic bacteria that might also impact cortisol-induced responses, and *vice versa* [116] (Chapter III [18]). Moreover, stress and stress-related hormones, such cortisol, are known to affect the lipid metabolisms in fish [117]. In this sense, the regulation of cortisol secretion by the tested additive could be underlying the previously mentioned intestinal transcriptional response, in which genes involved in the response to both lipids and steroid hormones were obtained along with the increase of bacterial sequences related with lipid metabolism (Chapter II [19]). Altogether, the described panoply of health-promoting benefits and their suggested crosstalk promoted by the tested phytogenics puts in evidence their wide range of action on distinct fish performance parameters and tissues, highlighting the complexity underlying their mode of action.

Providing insights into the mode of action of the tested phytogenics upon gilthead seabream mucosal tissues: A transcriptional complementary study approach

The original articles that comprise this thesis are some of the few available studies focused on aiming to decipher the mechanisms underlying the mucosal responses to the dietary supplementation of phytogenics. As reviewed in Chapter IV, most studies about the effect of phytogenics administration upon mucosal parameters only report variations in few biochemical markers, usually the same among studies, regularly obviating the core mechanisms mediating such physiological outcomes. Under this context, this thesis describes a holistic approach for evaluating the antiparasitic and antibacterial properties of a feed additive composed by microencapsulated carvacrol, thymol and garlic essential oil, as well as seeking to decipher its mode of action upon gilthead seabream mucosal tissues.

The overall evaluation of the tested feed additive upon each of the target tissues was determined by: 1) a microarray-based transcriptomic analysis of the mucosal tissue; 2) a functional enrichment analysis to identify classes of differentially expressed genes that may have an association with particular phenotypes and biological responses; and 3) the application of complementary methodologies in order to support and/or validate the molecular studies. The same microarray and bioinformatic tools were used for the transcriptional profiling of all the three studied mucosal tissues, which besides providing insights into the mode of action of the tested phytogenics upon each tissue, also allowed to evaluate from a comparative perspective the transcriptional profiling observed depending on each tissue considered. Aiming for this comparative approach, the samples of the three mucosal tissues studied were originated from the same common nutritional assay, and consequently, from fish reared under identical experimental conditions. Regarding the complementary methodologies used, these varied depending on the target tissue considered. For instance, a parasitic co-habitation challenge was applied for the gills' study –the main additive functionality aimed to be tested– as

well as a histochemical analysis of mucins produced by branchial mucous cells that could be contributing for the antiparasitic effect observed. Secondly, a microbiota analysis based on 16S rRNA sequencing was conducted to evaluate the potential effects of the tested phytogenics on the microbiota modulation and subsequent gut health, as one of the main performance indicators of animal welfare. Moreover, the antibacterial capacity of the skin mucus was evaluated in order to assess the protective effects of the tested feed additive against other potential pathogens, since skin is the main mucus-producing tissue, and one of the main routes of entrance for pathogenic bacteria into the organism. Altogether, these analyses performed in gills, intestine and skin showed that these three tissues positively responded to the dietary administration of the phytogenic-based additive, although some variations in the mucosal transcriptional responses were observed among the different tissues as it is discussed as follows.

The analysis of the transcriptomic profiling of the three studied mucosal tissues showed that most of the differentially expressed genes (DEGs) were up-regulated in fish fed the phytogenics-supplemented diet, whereas their modulation was consistently moderated in terms of fold change (FC) intensity, regardless of the studied tissue (*Figure 1*). The higher proportion of up-regulated genes associated to a moderate expression ($1<FC<1.5$) throughout the three studied mucosal tissues suggests a moderate and safe activation of the biological processes related to immunity that have been described and discussed along the Chapters I, II and III of this thesis. These changes in gene expression patterns were reported with no signs of compromising mucosal homeostasis. From a global point of view, 759 DEGs were obtained in the gills of fish fed the phytogenics-supplemented diet, of which 53 of those DEGs were also modulated in the intestine and another shared 40 DEGs were also modulated in the skin of fish fed the additive (*Figure 2*). In the intestine, a total of 581 DEGs were obtained, of which 53 were shared with the gills and 31 were modulated in the skin as well (*Figure 2*). Regarding the skin analysis, 534 total DEGs were obtained, 40 of them shared with the gills and 31 DEGS shared with the intestine (*Figure 2*). Surprisingly, only 5 DEGs were shared among the three tissues

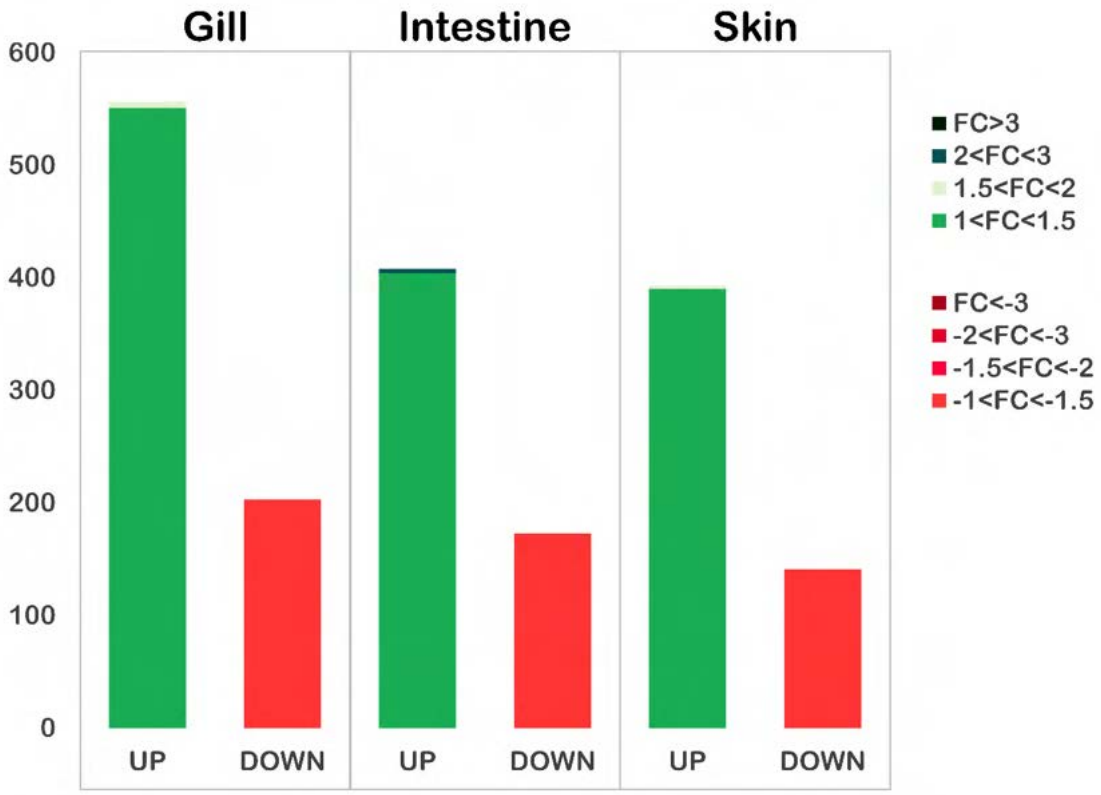


Figure 1. Number of differentially expressed genes (DEGs) in the gill, intestine and skin of gilthead seabream (*Sparus aurata*) fed a feed additive composed by garlic essential oil, carvacrol and thymol. The graph depicts the amount of up-regulated (green colors) and down-regulated (red colors) genes in all three studied mucosal tissues (unpaired t-test, $P < 0.05$). Most gene expressions were concentrated in the 1.0 to 1.5 fold change (FC) interval (up-regulated genes) and -1.5 to 1.0 FC (down-regulated genes).

analysis (*Figure 2*), evidencing tissue-dependent divergences in the mucosal responses with regard to the administration of the same phytogenic. From the overall 129 shared DEGs, only 67 genes were identified (*Figure 3*, *Table 1*), while the remaining DEGs were annotated as unknown genes. Unfortunately, one of the main constrains of the transcriptional analysis performed was the lack of fully annotated fish genes, particularly regarding the gilthead seabream genome, that allows to determine their identity and biological function. Under this context, missing pieces of the obtained transcriptional profiles could have brought added information on the fish mucosal responses to the dietary treatment. Nevertheless, the studies

included in this thesis have actively contributed to expand the transcriptome information of gilthead seabream, as well as providing a deeper understanding of mucosal-specific responses by means of dietary stimuli as further discussed.

Several factors may be conditioning the different transcriptional profiles obtained for each of the studied mucosal tissues, such as their intrinsic physiology and anatomy, the extrinsic environment that they are subjected to, and their specific microbiota composition [118]. Additionally, the potential degradation of the bioactive compounds along the digestive tract and their different pharmacokinetics within each target tissue may also affect their functionality, consequently exerting tissue-specific responses [119; 120]. Accordantly, most shared DEGs displayed different regulations between tissues (*Figure 3*). For example, several genes that were found to be up-regulated in gills were down-regulated in the intestine. The differential gene regulation observed in the intestine in comparison with the other tissues could be associated to an anti-inflammatory protective reaction of the intestine against the direct effect of the additive, since it is the inner mucosal tissue that interacts in first hand with the bioactive compounds and exogenous antigens present in the diet. In the gills and skin, this interaction with the phytogenic bioactive compounds may be occurring at a different level due to the compounds' metabolism and their potential alteration along the organism; thus, the immune transcriptional response could be expected to be accordingly different. Remarkably, the amount of the DEGs in the intestine was not substantially different from that observed in the other mucosal tissues (*Figure 1* and *2*), and particularly when compared with the skin, despite its distance from the absorption site of the phytogenics administered through the diet.

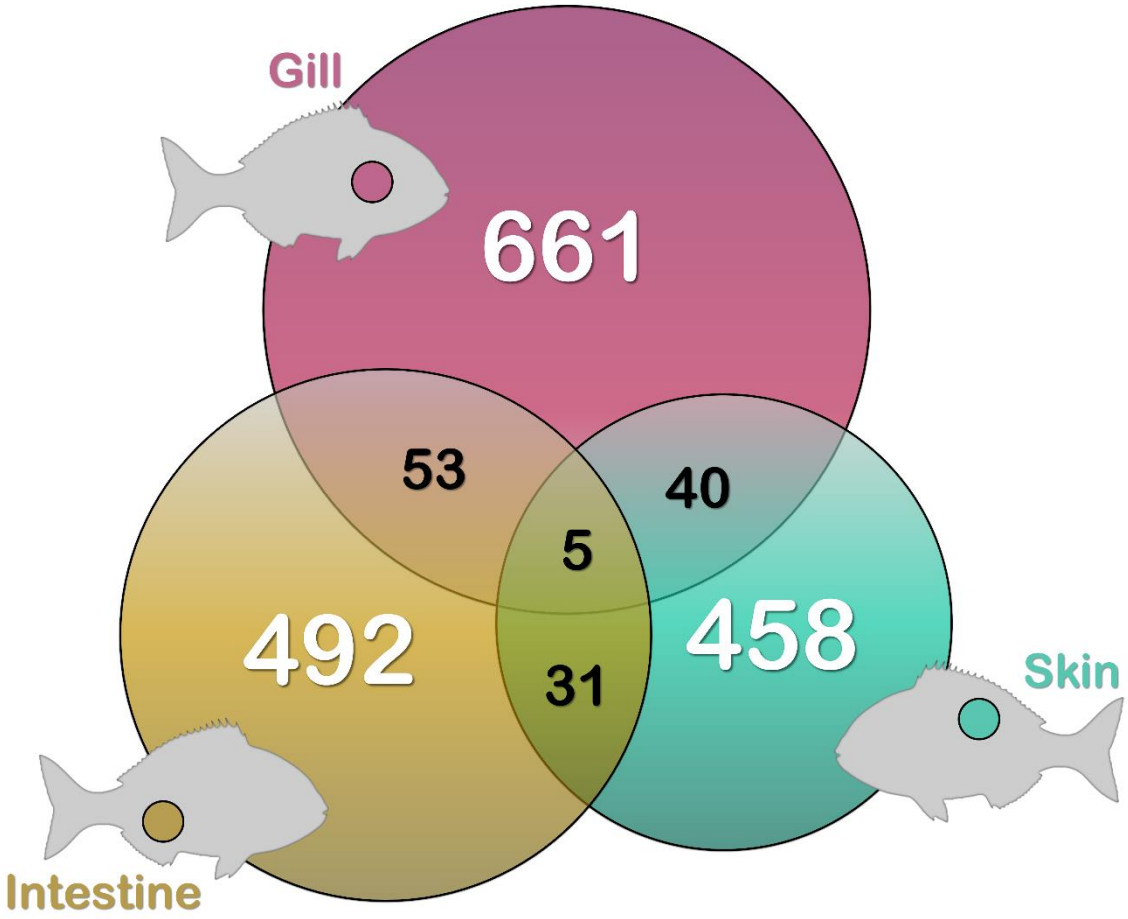


Figure 2. Venn diagram for representing the exclusive and common differentially expressed genes (DEGs) in the gill, intestine and skin of gilthead seabream (*Sparus aurata*) fed a feed additive composed by garlic essential oil, carvacrol and thymol (unpaired t-test, $P < 0.05$).

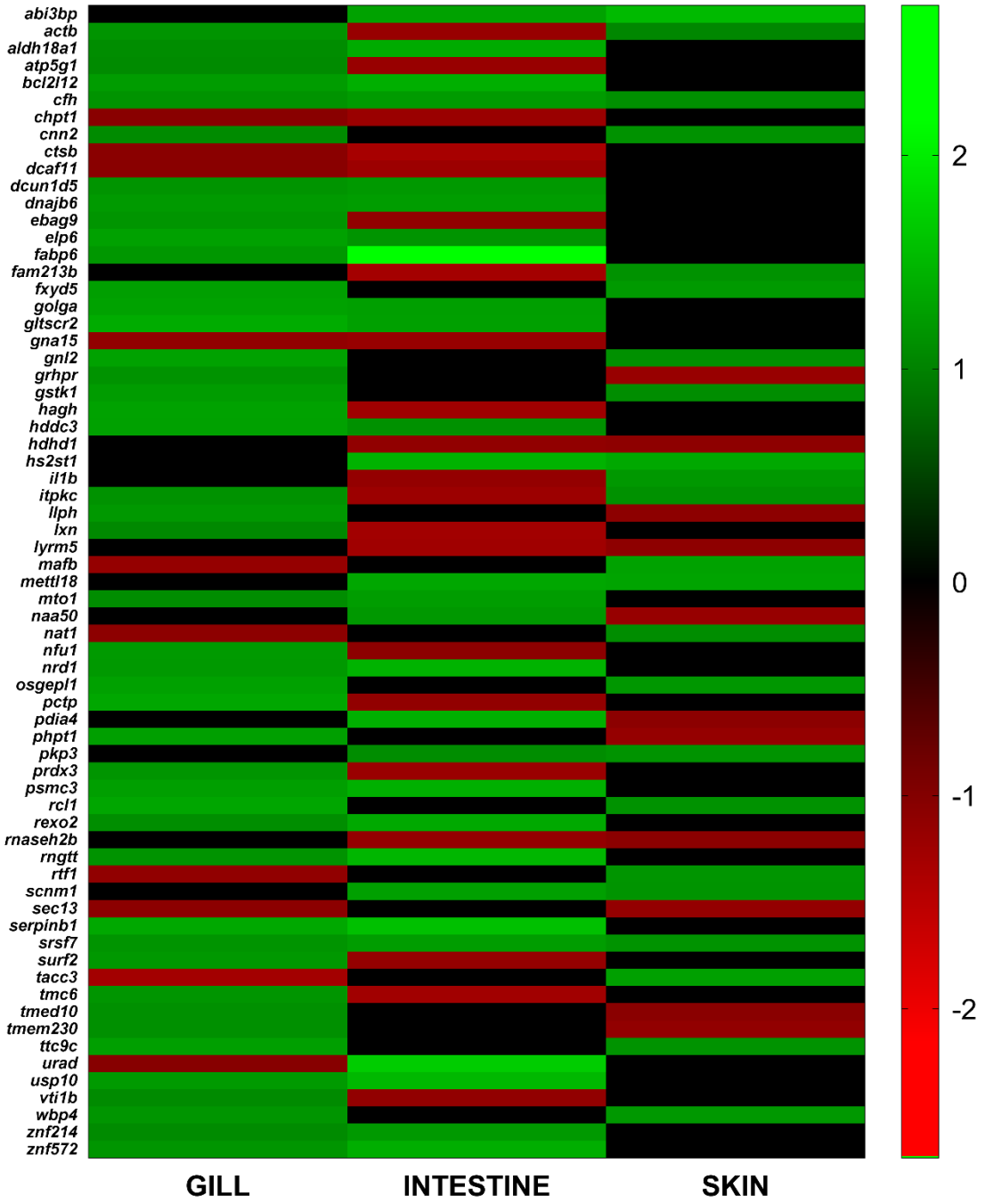


Figure 3. Differentially expressed genes (DEGs) shared between the gill, intestine and skin of gilthead seabream (*Sparus aurata*) fed a feed additive composed by garlic essential oil, carvacrol and thymol. The heatmap illustrates the regulation in terms of fold change (FC) intensity of the total shared DEGs (unpaired t-test, $P < 0.05$). Gene acronyms are referred in the left axis. Green: up-regulation; red: down-regulation; black: absence (color scale for FC values in the right axis).

Table 1. List of shared differentially expressed genes (DEGs) between at least two mucosal tissues including gills, intestine and/or skin of seabream (*Sparus aurata*) fed the garlic essential oil, carvacrol and thymol supplemented diet. Genes are alphabetically ordered. Gene description, respective acronym, fold-change intensity (FC) and modulation (green: up-regulation; red: down-regulation) are described.

		Gills	**Intestine**	**Skin**
Gene description	Acronym	FC	FC	FC
ABI Family Member 3 Binding Protein	*abi3bp*	*ns*	1.286	1.505
Actin Beta	*actb**	1.158	-1.201	1.048
Aldehyde Dehydrogenase 18 Family Member A1	*aldh18a1*	1.101	1.375	*ns*
ATP Synthase Membrane Subunit C Locus 1	*atp5g1*	1.072	-1.200	*ns*
BCL2 Like 12	*bcl2l12*	1.237	1.405	*ns*
Complement Factor H	*cfh**	1.152	1.219	1.119
Choline Phosphotransferase 1	*chpt1*	-1.069	-1.225	*ns*
Calponin 2	*cnn2*	1.079	*ns*	1.138
Cathepsin B	*ctsb*	-1.078	-1.340	*ns*
DDB1 And CUL4 Associated Factor 11	*dcaf11*	-1.061	-1.243	*ns*
Defective In Cullin Neddylation 1 Domain Containing 5	*dcun1d5*	1.161	1.204	*ns*
DnaJ Heat Shock Protein Family (Hsp40) Member B6	*dnajb6*	1.216	1.240	*ns*
Estrogen Receptor Binding Site Associated, Antigen, 9	*ebag9*	1.173	-1.151	*ns*
Elongator Acetyltransferase Complex Subunit 6	*elp6*	1.278	1.154	*ns*
Fatty Acid Binding Protein 6	*fabp6*	1.180	2.659	*ns*
Family With Sequence Similarity 213 Member B	*fam213b*	*ns*	-1.319	1.133
FXYD Domain Containing Ion Transport Regulator 5	*fxyd5*	1.258	*ns*	1.215
Golgin A7	*golga*	1.288	1.250	*ns*
NOP53 Ribosome Biogenesis Factor	*gltscr2*	1.381	1.263	*ns*
G Protein Subunit Alpha 15	*gna15*	-1.153	-1.186	*ns*

G Protein Nucleolar 2	*gnl2*	1.288	*ns*	1.121
Glyoxylate And Hydroxypyruvate Reductase	*grhpr*	1.149	*ns*	-1.197
Glutathione S-Transferase Kappa 1	*gstk1*	1.230	*ns*	1.097
Hydroxyacylglutathione Hydrolase	*hagh*	1.285	-1.272	*ns*
HD Domain Containing Protein 3	*hddc3*	1.273	1.130	*ns*
Pseudouridine 5'-Phosphatase	*hdhd1*	*ns*	-1.138	-1.120
Heparan Sulfate 2-O-Sulfotransferase 1	*hs2st1*	*ns*	1.440	1.344
Interleukin 1 Beta	*il1b*	*ns*	-1.167	1.192
Inositol-Trisphosphate 3-Kinase C	*itpkc**	1.142	-1.247	1.132
LLP Homolog, Long-Term Synaptic Facilitation Factor	*llph*	1.191	*ns*	-1.110
Latexin	*lxn*	1.051	-1.297	*ns*
Electron Transfer Flavoprotein Regulatory Factor 1	*lyrm5*	*ns*	-1.278	-1.117
MAF BZIP Transcription Factor B	*mafb*	-1.184	*ns*	1.288
Methyltransferase Like 18	*mettl18*	*ns*	1.330	1.306
Mitochondrial TRNA Translation Optimization 1	*mto1*	1.100	1.248	*ns*
N(Alpha)-Acetyltransferase 50, NatE Catalytic Subunit	*naa50*	*ns*	1.194	-1.187
N-Acetyltransferase 1	*nat1*	-1.103	*ns*	1.091
NFU1 Iron-Sulfur Cluster Scaffold	*nfu1*	1.216	-1.104	*ns*
Nardilysin Convertase	*nrd1*	1.200	1.443	*ns*
O-Sialoglycoprotein Endopeptidase Like 1	*osgepl1*	1.272	*ns*	1.182
Phosphatidylcholine Transfer Protein	*pctp*	1.332	-1.146	*ns*
Protein Disulfide Isomerase Family A Member 4	*pdia4*	*ns*	1.417	-1.100
Phosphohistidine Phosphatase 1	*phpt1*	1.257	*ns*	-1.176
Plakophilin 3	*pkp3*	*ns*	1.093	1.146
Peroxiredoxin 3	*prdx3*	1.165	-1.220	*ns*
Proteasome 26S Subunit, ATPase 3	*psmc3*	1.258	1.413	*ns*
RNA Terminal Phosphate Cyclase Like 1	*rcl1*	1.322	*ns*	1.141

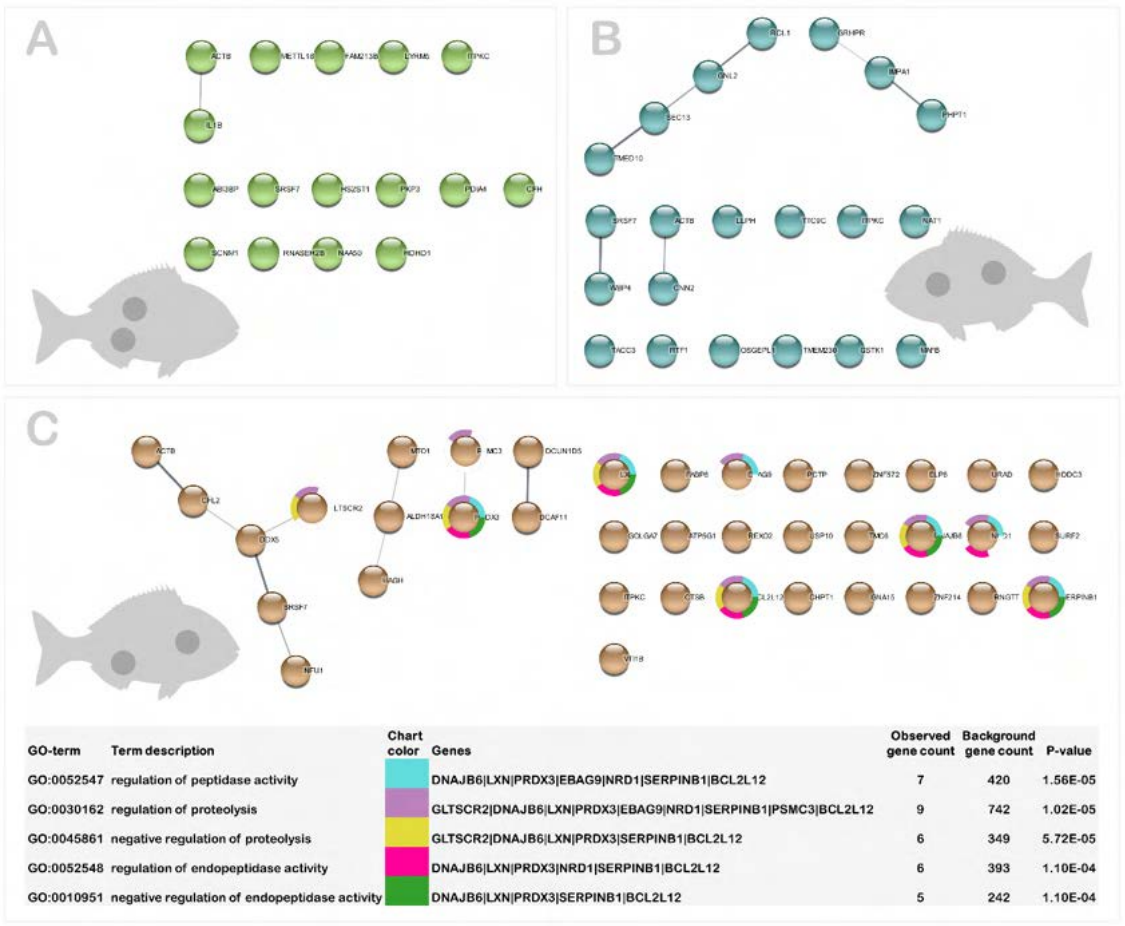
RNA Exonuclease 2	*rexo2*	1.103	1.349	*ns*
Ribonuclease H2 Subunit B	*rnaseh2b*	*ns*	-1.166	-1.083
RNA Guanylyltransferase And 5'-Phosphatase	*rngtt*	1.151	1.467	*ns*
RTF1 Homolog, Paf1/RNA Polymerase II Complex Component	*rtf1*	-1.141	*ns*	1.171
Sodium Channel Modifier 1	*scnm1*	*ns*	1.261	1.160
SEC13 Homolog, Nuclear Pore And COPII Coat Complex Component	*sec13*	-1.099	*ns*	-1.167
Serpin Family B Member 1	*serpinb1*	1.340	1.561	*ns*
Serine And Arginine Rich Splicing Factor 7	*srsf7**	1.165	1.247	1.144
Surfeit 2	*surf2*	1.190	-1.182	*ns*
Transforming Acidic Coiled-Coil Containing Protein 3	*tacc3*	-1.308	*ns*	1.262
Transmembrane Channel Like 6	*tmc6*	1.169	-1.284	*ns*
Transmembrane P24 Trafficking Protein 10	*tmed10*	1.121	*ns*	-1.076
Transmembrane Protein 230	*tmem230*	1.116	*ns*	-1.149
Tetratricopeptide Repeat Domain 9C	*ttc9c*	1.252	*ns*	1.145
Ureidoimidazoline (2-Oxo-4-Hydroxy-4-Carboxy-5-) Decarboxylase	*urad*	-1.050	1.683	*ns*
Ubiquitin Specific Peptidase 10	*usp10*	1.212	1.490	*ns*
Vesicle Transport Through Interaction With T-SNAREs 1B	*vti1b*	1.080	-1.149	*ns*
WW Domain Binding Protein 4	*wbp4*	1.168	*ns*	1.188
Zinc Finger Protein 214	*znf214*	1.077	1.201	*ns*
Zinc Finger Protein 572	*znf572*	1.167	1.389	*ns*

ns: not significant FC value
* highlights genes differentially expressed in the three studied mucosal tissues.

In this context, the gills were unexpectedly the tissue that revealed the highest number of DEGs promoted by the phytogenics-supplemented diet, rather than the intestine, which is the site of absorption of the feed additive and, *a priori*, the site where the major changes with regard to gene expression were expected (*Figure 1* and *2*). Although this is not clearly understood, this difference in tissue susceptibility may be a consequence of the combination of several factors, such as the gills particular functionality and the mode of action of the phytogenics bioactive compounds. As suggested in Chapter IV, it is possible that the terpenes and organosulfurs present in the studied feed additive display their mucosal immunomodulatory activity through the activation of TRP ion channels [121; 122; 123; 124]. In turn, the gills are one of the major organs conducting internal ionic regulation through specialized ionocytes, as they are actively involved in osmoregulation [125]. Consequently, after absorption and translocation through blood stream, the additive bioactive compounds may be acting significantly upon the highly irrigated gills, functionally regulating or amplifying specific ion fluxes, transporters and ionocytes, which might translate into a higher response to the administered compounds. This hypothesis opens interesting questions about at which extent gills may be susceptible to functional feed additives and recognized as promising target tissues for dietary therapeutic strategies. It is also important to take in account that the gut is constantly exposed to a vast array of foreign antigens present in food. Therefore, the intestinal mucosal immune system has evolved mechanisms in order to not only detect and eliminate pathogens, but essentially to avoid an exacerbated detrimental immune response to food antigens [126], which may explain the moderate response in comparison to the other tissues and why the intestine did not show major changes in gene expression when compared with the gills. Besides, since the transcriptomic methodology applied in our studies (i.e., transcripteractome) is not commonly used in the evaluation of feed additives, there is a gap in the available literature that limits the further exploring and discussing of current results with those from other studies.

Another aspect that could be influencing the gill's emphasized response to the phytogenics-supplemented diet is the fact that the section used for the RNA extraction in our study would hypothetically include the interbranchial lymphoid tissue (ILT). The ILT was originally described in the gill-associated lymphoid tissue (GIALT) of salmonids [127; 128; 129] and nowadays it is also recognized in several other teleost species [130]. The ILT is mainly characterized by aggregates of lymphoid cells, predominantly T cells, embedded in the epithelium, with few and scattered B cells and some strongly Mhc class II+ cells [127]. Although no comparable structure was identified in gilthead seabream so far (revealing the need for further research on teleost's mucosal-associated lymphoid tissues – MALTs), it is possible that the higher transcriptional immune response obtained from the gills with regard to the other mucosal tissues results from the sampling of the putative ILT (see methodology section of Chapter I [20]). If this were the case, the sampling of a tissue richer in lymphocytes than the other intestinal and skin sections sampled would have been reasonably translated into a more accentuated transcriptional immune response. In fact, in our study a gene coding for the C-C Motif Chemokine Ligand 4 (*ccl4*), which is a potent lymphocyte chemoattractant, was observed to be particularly up-regulated (FC = 1.366; P = 0.003; see Chapter I Supplementary Table 1) in the gills of fish fed the additive, suggesting a process of recruitment of immune cells in the gill's section evaluated. Moreover, in a previous gilthead seabream study, *ccl4* exhibited its highest expression levels in the gill and gut, while moderate levels were showed in other tissues, such the head kidney, spleen or thymus, and no expression in skin [131]. Interestingly, the referred study, besides clearly suggesting the key role of *ccl4* in mucosal tissues, puts in evidence the contrasting mucosal transcriptional response of each tissue in respect to their counterparts. Under this context, a deeper histological study of the mucosal tissues and the performance of an imaging immunodetection or flux cytometry analysis would have been critical for further characterizing the impact of the studied additive on the cellular community. Thus, further studies at cellular level are needed in order to fully understand the mode of action of the tested phytogenics.

In order to verify whether there are common biological processes responsible for common traits of the transcriptional profiles observed in the three studied mucosal tissues, an enrichment analysis of the previously mentioned shared DEGs among the three tissues (67 genes described in *Figure 3*, *Table 1*) was performed following the methodology described in Chapter I, II and III [18; 19; 20]. The different sets of shared DEGs did not revealed noteworthy interactions when merged and submitted to an enrichment analysis (*Figure 4*).



GO-term	Term description	Chart color	Genes	Observed gene count	Background gene count	P-value
GO:0052547	regulation of peptidase activity		DNAJB6|LXN|PRDX3|EBAG9|NRD1|SERPINB1|BCL2L12	7	420	1.56E-05
GO:0030162	regulation of proteolysis		GLTSCR2|DNAJB6|LXN|PRDX3|EBAG9|NRD1|SERPINB1|PSMC3|BCL2L12	9	742	1.02E-05
GO:0045861	negative regulation of proteolysis		GLTSCR2|DNAJB6|LXN|PRDX3|SERPINB1|BCL2L12	6	349	5.72E-05
GO:0052548	regulation of endopeptidase activity		DNAJB6|LXN|PRDX3|NRD1|SERPINB1|BCL2L12	6	393	1.10E-04
GO:0010951	negative regulation of endopeptidase activity		DNAJB6|LXN|PRDX3|SERPINB1|BCL2L12	5	242	1.10E-04

Figure 4. Protein–Protein Interactions (PPI) network for the differentially expressed genes (DEGs) shared between the skin and intestine (**A**), skin and gills (**B**), and gills and intestine (**C**) of gilthead seabream fed the garlic essential oil, carvacrol and thymol supplemented diet. Gene Ontology (GO) definitions, count of DEGs within each biological processes and respective false discovery rate are described in the graphical figure legend.

The fact that the modulation of genes that are differentially expressed in more than one of the studied tissues seems not to be connected and associated to specific biological processes corroborates that each mucosal tissue responds in a distinct and singular way to the dietary administration of the functional feed additive. Despite the few interactions, the genes shared between the gills and the intestine were observed to be enriched, participating in biological processes related to the regulation of proteolysis (*Figure 4C*). Regarding the genes' functionality, this regulation of proteolysis and peptidases biological processes seems to be particularly associated to mechanisms of cellular protection during immune responses in order to sustain cellular and tissue homeostasis and integrity. For instance, the Leukocyte Elastase Inhibitor Serpin Family B Member 1 gene (*serpinb1*), known to play an essential role in the regulation of the innate immune response through the regulation of neutrophils proteases proteolytic activity, was observed to be up-regulated in both gills and intestine of fish fed the additive-supplemented diet. Although these proteases are important for bacterial killing when released from neutrophils' granules, these potent enzymes may also cause detrimental effects upon host proteins, mainly under non-pathogenic circumstances, as it is the case of our sampling conditions. Thus, *serpinb1* up-regulation and consequent regulation of proteolytic activities protect cells and tissues from damage at inflammatory sites during stress and infection [132; 133; 134] or from the potential cytotoxicity of some immunostimulatory substances.

In addition, the mitochondrial Peroxiredoxin 3 gene (*prdx3*) was also enriched under the proteolysis context. The *prdx3* encodes a mitochondrial protein with recognized antioxidant functions [135] that has been used as a biomarker of stress response in fish [136; 137; 138]. The observed *prdx3* down-regulation in the fish intestine might indicate a decrease of the oxidative stress in the tissue and potentially, a positive impact of the tested additive on fish gut health, as suggested in Chapter II [19]. Conversely, *prdx3* was observed to be up-regulated in the gills of gilthead seabream fed the additive-supplemented diet. Interestingly, *prdx3* expression was observed to be significantly up-regulated in the gills of zebrafish in

response to *Aeromonas hydrophila* infection [139], demonstrating its relevance in the gill's immunity. The *prdx3* was also up-regulated in the head kidney of gilthead seabream resistant to the infection by the intestinal parasite *Enteromyxum leei* [140], suggesting the involvement of PRDX3 in effective antiparasitic immune responses; thus, also possibly playing a part in the antiparasitic response described in Chapter I [20]. Alternatively, the up-regulation of *prdx3* in gills might be a direct consequence of the lower parasite incidence in fish fed the phytogenics-supplemented diet, since *prdx3* down-regulation may result from fish gill's infection by trematodes parasites [141]. The pleiotropic nature of genes such *prdx3* highlights the difficulty of contextualizing the biological role of particular genes under a complex *in vivo* study; therefore, emphasizing the importance of the use of complementary analysis and/or challenges to corroborate the interpretation of the transcriptional responses observed.

Another up-regulated gene shared between the gills and the intestine that was observed to participate in biological processes related to the regulation of proteolysis is the BCL2 like 12 gene (*bcl2l12*). Interestingly, *bcl2l12* encodes for an anti-apoptotic factor whose overexpression in CD4+ T cells promotes the production of T helper (Th) type 2 cytokines in the intestinal mucosa [142]. Th2-type responses are widely recognized to predominantly arbitrate humoral immune responses against helminths [143]. In salmonids, external mucosal tissues, such gills and skin, present a Th2 biased immune environment protecting fish from external parasites and from damage by other inflammatory Th responses [144]. Moreover, neutrophils have been particularly suggested to act as potential effector cells responsible for Th2 initiation in response to helminth antigens [145]. In Chapter I and II [19; 20], gilthead seabream gills and intestine were described to present a similar enrichment in which the activation of acidophilic granulocytes was suggested to be one of the main mucosal responses to the phytogenics-supplemented diet. The specific up-regulation of *bcl2l12* in gills is of special relevance, since it is in agreement with the observed antiparasitic properties against the helminth ectoparasite *S. chrysophrii* reported in Chapter I [20]. Due to the

similitude between the gills and intestine's transcriptional responses in terms of enrichment analysis, which showed the modulation of leukocytes activation involved in immune responses (neutrophils degranulation), it would have been also interesting to evaluate this reported antiparasitic effect of the additive in the intestine by means of an enteric parasitic challenge. Until the analysis of the shared genes, the *bcl2l12* gene was unnoticed since it figured only in biological processes of proteolysis regulation in both gills (GO:0009987; GO:0051246) and intestine (GO:0009987; GO:0030162; GO:0052547; GO:0032269; GO:0052548), processes that by definition this do not intuitively point towards antiparasitic defense mechanisms. While enrichment analysis strategies allow retrieving the functional profiles of a gene set, they obviate the role of particular genes that could be key in specific responses; thus, these findings highlight the need to consider genes particular functionality in addition to their enrichment analysis.

According with the above-mentioned Th2-type response, the gene encoding the Transient Receptor Potential Cation Channel Subfamily M Member 4 (*trpm4*) was observed to be up-regulated (FC = 1.232; P = 0.018; see Chapter I Supplementary Table 1) in the gills of fish fed the functional additive. TRPM4 channels, in particular, are strongly expressed in CD4+ Th2 cells, regulating the Ca^{2+} levels within the cell, the motility and the production of Th2 cytokines [146]. This may suggest its involvement along with *bcl2l12* in the immunostimulatory effect of the tested phytogenic observed in gills. Moreover, the TRPV1, a receptor known to be activated by garlic derived organosulfur compounds [121; 147], was demonstrated to regulate Th2 immune responses in the airway mucosa of mice [148]. This may suggest an association between the TRP-induced response discussed in Chapter IV and a possible Th2-type anthelmintic response induced by the tested diet. In fact, the Ca^{2+} channels and Ca^{2+} influx are vital for T cell signaling processes, such as cytokine secretion, the consequent T cell activation and proliferation, as well as the T cell differentiation into Th effector cells [149]. The up-regulation of *il7* (FC = 1.25; P = 0.003; see Chapter I, Supplementary Table 1 in Firmino et al. [20]) and other interleukin receptors in gills promoted by the phytogenics-supplemented diet is in

accordance with the above-mentioned hypothesis. The cytokine IL-7 and its receptor, IL-7R, play an important role in mucosal immune responses since they are required for innate lymphoid cell development and maintenance, including T cells, and consequently for the generation of lymphoid structures and the support of the epithelial barrier defense [150], with the potential to act as Th2 responses enhancers [151]. In fish, IL-7 is for long recognized to be expressed in several tissues, including gills and intestine, and whose expression is positively modulated by immunostimulants [152]. In the particular case of gilthead seabream, those genes were observed to be predominantly up-regulated in fish challenged with the intestinal parasite *E. leei* [151], suggesting its involvement in antiparasitic responses. Altogether, these observations suggest that the modulation of Th2 immune responses amplified by TRP-activated leukocytes might underlie the antiparasitic effect observed in gills when fed the phytogenic-supplemented diet. Nevertheless, since the expression of genes coding for the traditional Th2 specific cytokines, IL-4, IL-5 and IL-13, were not observed to be significantly modulated under our current experimental conditions, further research is needed in order to clearly identify if the tested compounds are able to induce the particularly suggested TRPM4-induced Th2 response.

In line with the possible involvement of T cells regarding the observed transcriptional responses in both gills and intestine, the modulation of a set of identified immune-related genes in the skin was observed to be particularly involved in the regulation of T cells cytokines and T cell proliferation. These enrichment results were mainly associated to the up-regulation of the Major Histocompatibility Complex (MHC) Class II Alpha gene (*mhc-IIa*) and the Interleukin 1 Beta (*il1b*) in the skin of fish fed the phytogenics-supplemented diet. This putative T cell response in the skin could be resulting from the interaction with phagocytes as discussed in Chapter III [18]. Although no modulation of the *bcl2l12* or *trpm4* were detected in the skin, several genes coding for G-Proteins and G-protein Coupled Receptors (*gpr107*, *gnl2* and *gnb1*) were up-regulated in the skin of fish fed the phytogenics-supplemented diet. G-protein Coupled Receptors are key

players in many cellular and physiological processes, such as environmental sensing, neurotransmission, or endocrine and exocrine secretion, playing a central role in the development and activation of macrophages and in T cell-mediated immunity [153; 154]. They are also known to interact with TRP channels, an interaction described by the G-Protein Coupled Receptor – Transient Receptor Potential Channel Axis [155]. For instance, as it has been discussed in Chapter IV, it has been suggested that the effect of the activation of TRP channels by carvacrol, is enhanced by its co-activation with G-protein Coupled Receptors [122]. Regarding G-Proteins coding genes, *gna15* and *gnl2* were also observed to be differentially expressed in gills or the intestine of fish fed the additive. Therefore, the regulation of genes encoding G-Proteins and G-protein Coupled Receptors reinforces the already-mentioned idea that the mucosal immunomodulatory activity of the tested bioactive compounds present in the functional feed additive may result from the activation of TRP-related ion channels.

Regarding the five DEGs shared between the three studied mucosal tissues, four were identified (Table 1). Among them, the Inositol-Trisphosphate 3-Kinase C (*itpkc*) gene was up-regulated in the gills and the skin of fish fed the phytogenics-supplemented diet, while it was down-regulated in the intestine. The *itpkc* encodes a kinase that has a regulatory role in Ca^{2+} responses to extracellular signals, which in higher vertebrates is known to act as a negative regulator of T cell activation [156]. Although *itpkc* role in teleosts is poorly described and understood, its modulation by the phytogenics-supplemented diet suggests its involvement in i) Ca^{2+} fluxes, and ii) the activation and regulation of the cellular immune response, both promoted by the additive bioactive compounds. However, based in the existent literature, the accurate biological significance of the differential regulation of this gene in the studied tissues remains unclear. Moreover, the absence of measurements of the teleost mucosal immunoglobulin IgT/IgZ, as a marker that evidences the mounting of a further adaptive immune response mediated by B lymphocytes, detracts further assumptions about the involvement of immune cells based exclusively in gene regulation.

The Actin Beta (*actb*) gene was also observed to be up-regulated in both gills and skin, while it was down-regulated in the intestine. Actins are highly conserved proteins ubiquitously expressed in all eukaryotic cells that are involved in several processes such cell crosstalk and division, gene transcription, motility and contraction and repair of damaged DNA [157; 158]. Regarding fish, actin is commonly found in the mucus of several fish species, including gilthead seabream [159; 160; 161; 162; 163; 164], which has led to speculations on its immune function in fish defense, as discussed in Chapter III [18]. For instance, ACTB levels were observed to be significantly increased in the epidermal mucus of Atlantic salmon challenged with sealice [165; 166]. In gilthead seabream, the presence of ACTB in skin mucus was also previously reported to be modulated by dietary treatments [164]. In insects, the extracellular cytoplasmic actin was observed to bind to bacteria surface, mediating their phagocytosis and killing [161]. In fact, phagocytic events are particularly driven by rearrangements of the actin cytoskeleton [167]. Therefore, the association of increased phagocytic processes to the *actb* modulation in the skin of fish fed the functional feed additive was also suggested in Chapter III [18]. In addition, the actin cytoskeleton is a key regulator of the mucosal barrier integrity due to its involvement in the assembly and remodeling of epithelial cell junctions [168]. Under this context, *actb* up-regulation by the phytogenics-supplemented diet in the gills and skin could prove to be beneficial. On the other hand, its down-regulation in the intestine may be attributed to lower epithelial turnover rate associated with a better health gut condition. Nevertheless, although there is for long evidence that actin expression may vary depending on biochemical stimuli, *actb* is usually selected as reliable reference gene for the quantification of mRNA (qPCR) [169], and, therefore, its regulation is difficult to interpret based in the existent literature. Since *actb* is differentially expressed among the three evaluated tissues, our results support either its significant involvement in the mucosal protection and integrity, and the concern that its use as a stable and constitutive reference gene should be carefully revised when evaluating different tissues.

Another gene shared among the three studied mucosal tissues whose transcripts were recurrently observed to be increased by the phytogenic-supplemented diet is the Complement Factor H (*cfh*). CFH is a glycoprotein with a major regulatory role in the complement system activation that responds to pathogen-associated molecular patterns (PAMPs) and increases the contact between neutrophils and pathogens, increasing cell's phagocytosis capacity and antimicrobial activity [170; 171]. CFH is also reputed for protecting host cells and tissues from the self-innate immunity [172]. Although in fish CFH is predominantly expressed in the liver compared to other tissues and organs like the muscle, intestine, fins, eyes, and gills [170], present data indicated that *cfh* may also play a relevant role in the regulation of the alternative pathway of complement in mucosal tissues. In accordance, several complement factors have been previously identified in fish mucus secretions [165; 166], including gilthead seabream [159; 164]. Moreover, it has been shown that alternative complement activity increases in the serum of gilthead sea bream when exposed to the *E. leei* [173]. However, the expression of complement-related genes is usually down-regulated in the intestine of those exposed fish [174; 175]. Thus, complement expression and/or activity is affected by pathogen exposure and may vary between systemic *vs.* mucosal responses [176]. As reviewed in Chapter IV, several studies evaluating the effects of phytogenics derived from Lamiaceae family or *Allium* sp. of aromatic plants in mucosal tissues have reported an increase in complement levels or activity, corroborating the intervention of complement factors in phytogenic-induced mucosal responses. In fact, the regulation of the complement plays an important role in the functional plasticity of the intestinal epithelial response to different nutritional, microbial, and chemical challenges [177]. Thus, the collection of these data highlights the relevance of the complement system as a key player in the mucosal immunostimulation induced by phytogenics.

The last but not least, the up-regulation of the Serine and Arginine Rich Splicing Factor 7 (*srsf7*) gene was also observed among the three studied MALTs. Splicing factors such SRSF protein kinases are required for transcription, mRNA splicing and mRNA export from the nucleus [178]. Accordingly, RNA metabolism and gene

expression related processes were consecutively obtained from the enrichment analysis of the different mucosal tissues, which suggests that gene transcription and protein biogenesis machinery are promoted under such dietary conditions. In this sense, these results may be correlated, at least in part, with one of the main characteristics of the mucosal tissues: the production of mucus. In fact, the histochemical analysis of the mucins from gills demonstrated an increase in acidic glycoproteins containing sialic acids as well as a hypertrophy of the mucous cells in fish fed the additive-supplemented diet, indicating a potentiation of mucins secretion and renewal, boosting its protective function as discussed in Chapter I [20]. In gills, the up-regulation of the gene coding for the O-sialoglycoprotein Endopeptidase Like 1 (*osgepl1*) was also suggested to be involved in antiparasitic effect described in Chapter I [20] due to its role in the proteolytic mucin degradation, which is a characteristic of the host glycoproteins "shedding" defense mechanism. Similarly, *osgepl1* up-regulation was also observed in the skin of fish fed the additive, which might indicate changes in the histochemical properties of the skin mucins and/or epithelial cells. Unfortunately, such histochemical study was not performed. Conversely, the histological analysis of the intestinal mucosa did not reveal significant differences in the histochemical properties of intestinal mucins nor in the number of goblet cells between dietary treatments (33.5 ± 6.4 cells/mm of basal lamina; $F = 0.68$; $P = 0.691$; *Appendix I*, unpublished data), which was associated to the high inter-individual variability observed within each experimental group. Furthermore, the presence of a large number of eosinophilic granulocytes at the level of the intestinal submucosa was also observed (*Appendix II*, unpublished data), even though this is a common feature of the gilthead seabream intestine (C. Sarasquete, personal communication) rather than a dietary effect. Since, no apparent significant differences were detected regarding the infiltration levels of eosinophilic granulocytes, the observed regulation of immune related biological processes associated with granulocytes activation in particular, are probably not associated to a higher number of immune cells *in situ* under healthy conditions. As outlined before, a deeper histological analysis of the mucosal

tissues or a study of the cellular communities would have provided answers for remaining interrogations about the impact of the studied additive.

Enrichment analysis tools are applied in order to facilitate the interpretation of biologically meaningful data, such the simultaneous analysis of several genes under certain biological condition [179]. Under this context, it was observed that in the particular case of the skin enrichment analysis only one single biological process gene ontology (GO) was significantly enriched among the experimental diets: the RNA processing biological process (GO:0006396); contrarily to the observed for the remaining mucosal tissues studied. If we consider that the number of genes related with RNA processing is considerably higher than the number of genes related with immunity, it is mathematically logic that immune-related processes may be hidden among all the data. This lack of biological processes enrichment led to the revision and complementation of the skin transcriptional analysis, in which other enrichment elements were taken in account, such as cellular components and KEGG pathways, as described in Chapter III [18]. Therefore, in order to evaluate in depth and contextualize the results obtained from the skin and mucus analysis, an emphasis was placed on a selected set of immune-related genes. In this case, the transcriptomic analysis was based in three main criteria: i) the statistical criterion ($P < 0.05$, GO enrichment analysis); ii) the biological criterion, where genes were selected based on their definition and function according to the available literature; and iii) the representation of networks that encompassed the overall transcriptional results. The biological significance of the identified immune-related genes were further connected to the results of the *in vitro* challenge with pathogenic bacteria in the skin mucus, in which the secretion of immune-related components into the mucus was suggested in Chapter III [18]. Although a relation between mucosal tissue metabolism and its exuded mucus can be assessed using proteomic approaches [180], no proteomic analysis was performed in the present study in order to further evaluate in which extent the additive could be actually affecting the mucus composition. Nonetheless, the *in vitro* results confirmed the positive effect of the phytogenic evaluated on the antibacterial properties of the skin mucus.

This dissimilarity among the results obtained from the skin in respect to gills and/or the intestine, evidences differences in the immunostimulatory mechanisms underlying the different mucosal tissues. On one hand, being the skin the largest mucus producer tissue in fish, it is possible that the differences observed are a consequence of the preponderance of DEGs related with transcription and protein synthesis and secretion [18; 180]. On the other hand, it has been demonstrated that the secretion activity of teleost plasmablast, a major lymphocyte population in some mucosal surfaces, in the gills and gut is not comparable to that of the skin [181]. This denotes different plasmablast's homing requirements among mucosal tissues, possibly due to the skin particular microbiota that do not support the homing and/or survival of some lymphocytes [181]. These findings would be in accordance with the lack of enrichment processes related to leukocytes activation in the skin of fish fed the phytogenics-supplemented diet. In fact, there are significant differences in microbiota beta-diversity between the two outer mucosal tissues, gills and the skin, in gilthead seabream [182] with only 17% to 19% of shared amplicon sequence variant (ASVs) reported between gill and skin microbiota of farmed seabream [183], evidencing significant divergence between the microbiota of each tissue. Thereafter, the distinct commensal microbiota composition may be also responsible for the observed modulation of the mucosal tissues gene expression and *vice versa*, although this subject is still poorly explored in fish, particularly in the skin [184].

While no skin or mucus microbiome analysis were performed in the present study, the observed mucus inhibitory effect against the two pathogenic bacteria *V. anguillarum* and *P. aguilliseptica* promoted by the tested additive described in Chapter III [18], could be hypothetically affecting the skin microbial composition. The adherence of bacteria to mucosal surfaces and the potential infection by pathogenic strains depend on mucus characteristics and composition [185; 186], which according to our results seems to be considerably affected by the feed additive. Both *Vibrio* and *Pseudomonas* genera are present in the skin as commensal microbiota in farmed gilthead seabream [182], co-existing in the host mucosal surfaces that sophistically control their abundance in order to avoid dysbiosis that

could ultimately lead into bacterial infection [187]. Under this context, the mucus inhibitory capacity against bacterial pathogens induced by the additive could be potentially altering the skin microbiota composition, which in turn might be also contributing to the transcriptional regulation observed. In effect, the modulation of the host-microbiota co-metabolism through the crosstalk between gut and bacteria in the inflammatory regulation due to the dietary administration of the feed additive is suggested in Chapter II [19]. Despite the lack of data on this subject, a similar response would be expected for both skin and gills. Furthermore, the observed affectation of the bacterial growth in the skin mucus in fish fed the phytogenics-supplemented diet may also suggest the efflux of the additive bioactive compounds through the skin into the mucus, as proposed in previous studies [108; 188], possibly exerting a direct inhibitory effect on the cultured bacteria.

Future academic and industrial research perspectives

Although the results from the present thesis have provided insights into the mode of action of the tested phytogenics and some of the potential mechanisms underlying their immunostimulatory properties upon gilthead seabream mucosal tissues, several interesting questions have arisen from the research described that need further attention. For instance, the analysis presented in this thesis only considered the evaluation of the effect of the tested feed additive in the physiological response of mucosal tissues. However, the assessment of its impact upon other non-mucosal tissues and/or the systemic immunity is of great importance in order to fully characterize the implications of the administration of carvacrol, thymol and garlic essential oil on the overall health status of gilthead seabream.

In addition, a particularly interesting line of research would be the evaluation of the activity of the bioactive compounds present in the tested additive in fish biological fluids, such as blood and mucus, which may potentially explain tissue-specific responses. Under this context, the studies described in this thesis have

demonstrated that the dietary administration of bioactive compounds promotes physiological responses in other tissues far from their site of absorption, which clearly indicate a translocation of these bioactive compounds throughout the organism. However, at which extent these compounds are metabolized, transported or degraded in different body compartments is unknown; thus, the evaluation of the presence of related metabolites in biological fluids and/or tissues may provide some insights about the magnitude of their pharmacological properties. Since pharmacokinetic studies of these particular compounds are scarce and even inexistent in fish, more efforts should be made to elucidate the mode of action of these compounds when administered individually or in a blend in order to develop further promising synergistic formulas for supporting fish health and welfare.

Another fascinating research line that could provide answers to the remaining questions derived from this study on the functionality of the tested feed additives is the in-depth study of the compounds-host-microbiota interactions. In the present thesis, this approach was only used for the intestine, where microbiota is widely recognized to interact with both feed and host metabolism. However, such interactions in other tissues characterized to cooperate with microorganisms, such as the skin and gills, are of equal significance and therefore the evaluation of those interactions that may be modulated by dietary strategies are quite relevant. In this context, exploring holistic approaches in which all different organs and systems, including microbiota from different body regions, as well as combining *in vitro* or *ex vivo* tools, may be of value for providing insights into the complex mode of action of the bioactive compounds included in the tested additive.

The applied component of this research is perhaps the most outstanding objective of this doctoral thesis. Besides solving scientific questions, the overall results and added knowledge obtained from this industrial thesis aimed to drive the development and consolidation of new nutritional tools that may be used to hamper the initially referred prediction of emergent disease outbreaks related to the growth in intensive production systems and the increasing pressure of climate change. Under this context, a fundamental aspect for the further development and

application of the tested additive in the aquaculture sector is the need for its testing in other aquaculture-relevant fish species and under additional challenges, such as the evaluation of the effectiveness of the tested additive against other parasites, bacterial and viral infections, stress and/or dietary challenges. From the industrial perspective, field validations under actual farming environments are critical for the corroboration of the results obtained under the present lab-experimental conditions and for the establishment of the final product in the aquaculture value chain. In this context, the studied additive and its variants are currently being tested in other fish species in field trials. Additionally, although the tested additive composition, inclusion level and period of administration were established according to the existent literature and the industrial know-how, further studies assessing optimized formulations and supplementation protocols (i.e., minimum administration period for guaranteeing its effectiveness, adjustments to seasons of the year, basal diet formulation, etc.) according to different farming scenarios are required. In synthesis, the possibilities for the development and improvement of the tested phytogenics-based additive in the aquaculture industry and for the opening of new research lines are endless.

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CONCLUSIONS

CONCLUSIONS

1. The administration of a microencapsulated feed additive at 0.5%, containing garlic essential oil, carvacrol and thymol, in on-growing gilthead seabream (*Sparus aurata*) promoted the mucosal innate immunity without compromising fish growth parameters.

2. At gills' level, the administration of the tested phytogenic had an antiparasitic protective effect against *Sparicotyle chrysophrii*. This phytogenic promoted a significant reduction of 78% in the *S. chrysophrii* total parasitization abundance, and a decrease in the prevalence of most parasite's developmental stages.

3. The transcriptomic analysis performed by microarrays of the gills of fish fed the phytogenics-supplemented diet showed a predominant up-regulation of genes related to biogenesis, vesicular transport and exocytosis, leukocyte-mediated immunity, oxidation–reduction and overall metabolism processes. These genes were related to a tissue-specific pro-inflammatory immune response arbitrated by degranulating acidophilic granulocytes, which was also sustained by antioxidant and anti-inflammatory responses.

4. The histochemical study of gills showed an increase in acidic carboxylated glycoproteins containing sialic acid in mucous and epithelial branchial cells of fish fed the phytogenics-supplemented diet, suggesting the promotion of the mucosal defence mechanism through the modulation of gills' mucin secretions.

5. The tested functional diet appears to promote the intestinal local immunity through the impact of the tested phytogenics on the host-microbial co-metabolism, and consequent regulation of significant biological processes in the intestinal mucosa. Both genes' transcripts and bacterial sequences revealed correlated functionalities, evidencing the crosstalk between gut and microbiota in the intestinal response observed.

6. The transcriptomic enrichment analysis of the mid-anterior intestine of gilthead seabream fed the phytogenics-supplemented diet showed the regulation of genes related to processes of proteolysis and inflammatory modulation, immunity, transport and secretion, response to cyclic compounds, symbiosis, and RNA metabolism.

7. The activation of leukocytes, such as acidophilic granulocytes, was suggested to be the primary actors of the innate immune response in the intestine promoted by the tested functional feed additive in the gut.

8. The 16S Ribosomal RNA (rRNA) sequencing of the anterior and posterior sections of the intestine of gilthead seabream revealed that the phytogenics-supplemented diet promoted subtle, but significant alterations in bacterial abundances in terms of phylum, class and genus. However, no effects on the alpha biodiversity values were obtained.

9. Changes in microbiota composition, such as the decrease in *Bacteroidia* and *Clostridia* classes, were suggested to participate in the modulation of the intestine transcriptional immune profile observed in fish fed the functional diet. The analysis of the intestinal microbiota functionality revealed an increase in bacterial sequences associated with glutathione and lipid metabolisms, suggesting metabolic modifications that could be potentially affecting the observed immune-related transcriptional response.

10. At the skin mucosa level, the tested phytogenics reduced both *Vibrio anguillarum* and *Pseudomonas anguilliseptica in vitro* growth capacity in the skin mucus of gilthead seabream fed the functional feed additive, suggesting an enhanced skin mucus inhibitory capacity against bacterial fish pathogens.

11. The enrichment analysis of the skin transcriptional profile of gilthead seabream fed the phytogenic-supplemented diet revealed the regulation of genes associated to cellular components involved in the secretory pathway, such as endolysosomes and phagosomes, suggesting the stimulation and recruitment of phagocytic cells.

12. Immune and epithelial cells' activation in mucosal-associated lymphoid tissues (MALTs) are hypothesized to be conducted by means of transient receptor potential (TRP) cation channels mediated by phytogenics' bioactive compounds.

13. The tested functional feed additive decreased the levels of cortisol in mucus, indicating a reduction in the fish allostatic load due to the properties of the tested additive.

14. Altogether, the dietary supplementation of garlic, carvacrol and thymol is demonstrated to promote the gilthead seabream mucosal innate immunity and the mucus protective capacity, decreasing the mucosal tissues susceptibility to be infested by parasites and colonized by pathogenic bacteria. This protective effect did not compromise the somatic growth or microbiota homeostasis. Therefore, this strategy appears to be an effective and safe tool to be used in functional diets for aquaculture.

15. Through the analysis and the in-depth technical characterization of the additive studied based in the technology and knowledge transfer, this thesis generated a set of positive and comprehensive results that allows the

placement of the product on the market and, consequently, its application in real aquaculture industry context.

APPENDICES

APPENDIX I

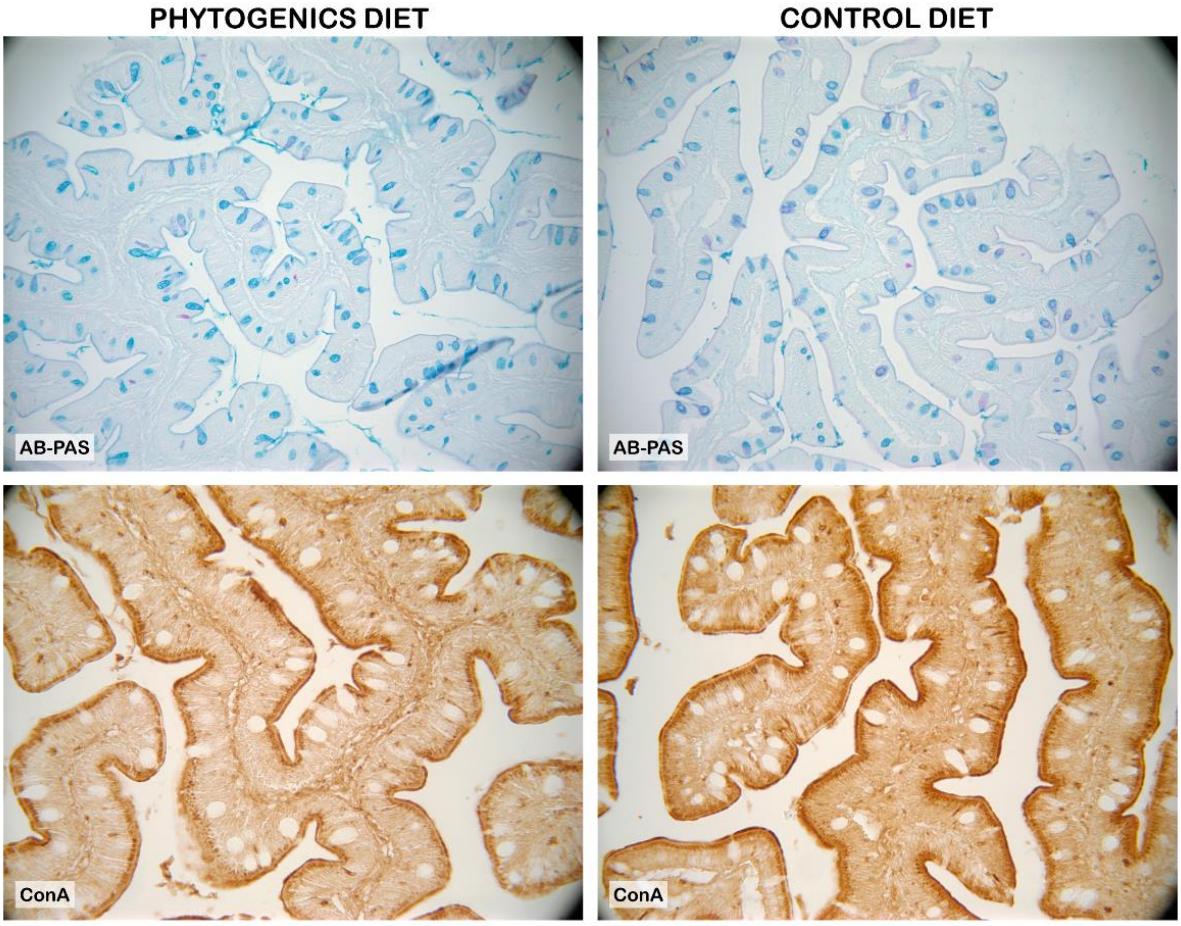
Appendix I. Histochemical properties of the intestinal mucous cells of gilthead seabream fed the control diet and the diet supplemented with a blend of garlic essential oil, carvacrol and thymol. Results are expressed as the semiquantitative assessment of colour intensities by the scores of four independent observers: (0) negative; (1) weak; (2) moderate; (3) intense; and (4) very intense (See Supplementary Information in Firmino et al. 2020, available at https://doi.org/10.1038/s41598-020-74625-5). PAS - Periodic Acid Schiff, AB - Alcian Blue, ConA - Concanavalin A, WGA - Wheat germ agglutinin, SBA - Soybean agglutinin, SNA - *Sambucus nigra* lectin.

Diet	Control diet	Phytogenics diet
General histochemistry		
PAS	1-3[2]	1-3
AB pH 0.5[1]	1-3	1-3
AB pH 1.0[1]	1-3	1-3
AB pH 2.5[1]	1-3	1-3
Lectin histochemistry		
ConA[4]	0-1	0-1
WGA[1]	0-3	0-3
SBA	0-3	0-3
SNA	0-3[5]	0-3[5]

[1] most cells intensity 3;
[2] most cells intensity 2-3;
[3] most cells intensity 1-2;
[4] most cells intensity 0;
[5] positive reaction in most cells.

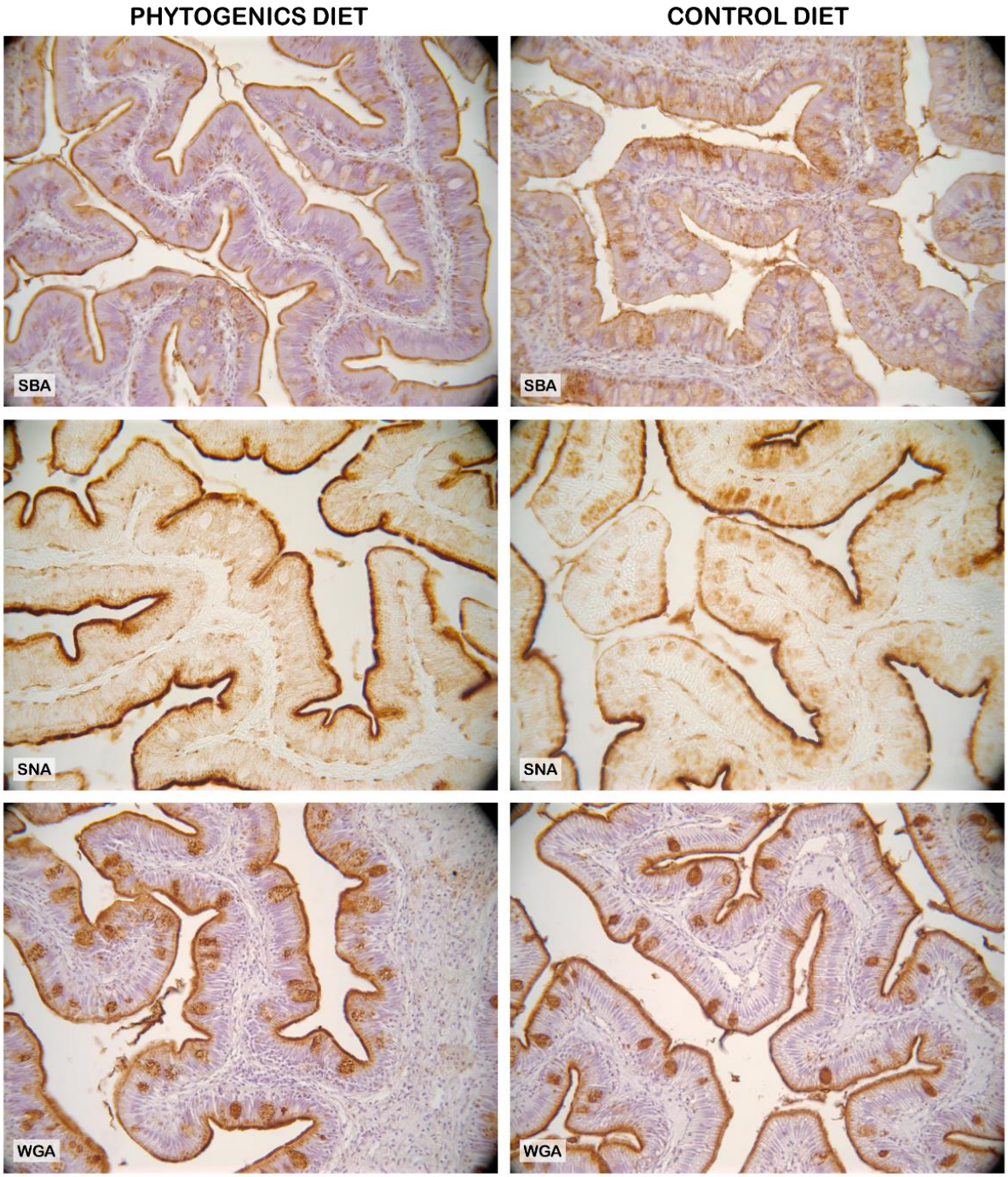
APPENDIX II

Appendix II. Histological sections from the intestine of gilthead seabream (*Sparus aurata*) fed the control diet (right column) and the diet supplemented with a blend of garlic essential oil, carvacrol and thymol (left column). High presence of neutral glycoproteins in intestinal mucous cells from control and phytogenics-supplemented diets (staining: AB-PAS). Most mucosal cells stained blue (carboxylated groups) or violet (combination of neutral and acid mucins) while very few stained red (neutral mucins). In general, a negative or low reactivity to Concanavalin A (mannose residues) is observed (staining: ConA lectin). Regarding the SBA lectin, which indicates the presence of galactose and N-acetyl galactosamine, the reaction was variable among individuals (staining: SBA lectin). The SNA lectin reaction, indicating the presence of sialic acid, was positive in most mucous cells (staining: SNA lectin). Most cells show high reactivity to the WGA lectin indicating the presence of N-acetylglucosamine and sialic acids (staining: WGA lectin).



(continued)

Appendix II. *(continued)*



ABSTRACT

Aquaculture growth will unavoidably involve the implementation of innovative and sustainable production strategies, being functional feeds among the most promising ones. A wide spectrum of phytogenics, particularly those containing terpenes and organosulfur compounds, have been gaining increasing interest in aquafeeds, due to their growth promoting, antimicrobial, immunostimulant, antioxidant, anti-inflammatory and sedative properties. Although the impact of phytogenics upon fish mucosal immunity has been extensively evaluated, most of the studies fail in addressing the mechanisms underlying their pharmacological effects. Under this context, the set of studies gathered in this thesis aims to provide insights on how fish mucosal tissues are immunomodulated by phytogenics, in particular by the administration of a microencapsulated feed additive composed by garlic essential oil, carvacrol and thymol. A holistic approach integrating both key performance indicators and the transcriptional immune response of three selected mucosal tissues – gill, intestine and skin – was applied in one of the most important marine fish species farmed in the Mediterranean Sea, the gilthead seabream (*Sparus aurata*). The overall analysis of the results indicated that the dietary supplementation of garlic, carvacrol and thymol promote the gilthead seabream mucosal innate immunity and the mucus protective capacity, decreasing the mucosal tissues susceptibility to be infested by parasites and colonized by pathogenic bacteria, without compromising somatic growth or microbiota homeostasis. Therefore, the strategy evaluated is an effective and safe tool to be used in functional diets for aquaculture. Besides solving scientific questions, the overall results and added knowledge obtained from this industrial thesis aim to drive the development and consolidation of new nutritional tools that may be used to hamper emergent disease outbreaks related to the growth in intensive production systems and the increasing pressure of climate change.

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Always wondering, her path has been shaped by her love for nature and her belief in a fairer future for humankind and our planet.

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