

# Alternative Proteins for Fish Diets

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Aquaculture has been challenged to find alternative ingredients to develop innovative feed formulations that foster a sustainable future growth. Given the most recent trends in fish feed formulation on the use of alternative protein sources to decrease the dependency of fishmeal, it is fundamental to evaluate the implications of this new paradigm for fish health and welfare.

Keywords: aquaculture ; fish nutrition ; fish diet formulations ; fishmeal replacement ; alternative protein sources

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## 1. Impacts of Alternative Protein Sources on Fish Intestinal and Hepatic Health

In the past two decades, an intense research effort has been devoted to unlocking the potential of plant-derived ingredients, as an alternative to gradually replace traditional ingredients of marine origin (fishmeal and fish oil), in aquafeeds formulation <sup>[1][2]</sup>. Earlier, soybean was considered a good alternative to fishmeal, due to its high protein content, relatively balanced amino acid profile, and availability <sup>[3]</sup>. However, the first reports showed that dietary inclusion of soybean meal at levels above 10% induced enteritis in salmonids <sup>[4]</sup>. The disturbance was associated with the presence of antinutritional factors in crude soybean meals, especially soya saponins <sup>[5]</sup>. High levels ( $\pm 20\%$ ) of dietary soybean meal inclusion are still reported to induce enteritis in Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) <sup>[6][7][8]</sup>; further research has shown that salmonids were not the only fish affected. Signs of soybean-induced enteritis were also recently reported in orange-spotted and hybrid groupers (*Epinephelus coioides* and *E. fuscoguttatus*  $\times$  *E. lanceolatus*) <sup>[9][10]</sup>, yellowtail (*Seriola dorsalis*) <sup>[11]</sup>, Japanese seabass (*Lateolabrax japonicus*) <sup>[12][13]</sup>, and totoaba (*Totoaba macdonaldi*) <sup>[14]</sup>.

The integrity of the intestinal epithelium is fundamental to secure nutrient absorption, and the negative effects of plant protein-based diets in the intestinal health may lead to disturbances in the intestinal absorptive function. In meagre (*Argyrosomus regius*) juveniles that were fed a diet containing more than 50% of plant ingredients for 10 weeks, the intestinal epithelium showed a secretory function, instead of its normal absorptive function, indicating a pathological state of diarrhoea <sup>[15]</sup>. This intestinal disturbance has been previously reported in Atlantic salmon presenting soybean-induced enteritis <sup>[16][17]</sup>. The alterations in the intestinal epithelium, due to enteritis, negatively affect nutrient digestibility, intestinal enzyme activities, and the expression of amino acid and peptide transporter genes <sup>[12][14]</sup>. Therefore, the disturbances found at intestinal epithelial level are translated in a lower nutrient availability for fish, which, in some circumstances, may be reflected in a reduction in growth performance.

Besides the important role of the intestine in nutrient absorption, intestine integrity is essential in the defence against pathogens. Japanese seabass, when fed soybean-based diets (>30% of inclusion) for eight weeks, showed increased intestinal permeability and impaired intestinal mucosal barrier function <sup>[12]</sup>. Soya saponins, per se, have been shown to increase intestinal epithelial permeability in Atlantic salmon after feeding fishmeal-based diets with soya saponin inclusion for 53 days, even if not inducing enteritis <sup>[16]</sup>. It should be noted that not only soybean-based diets induce disturbances at intestinal level. Diets with high levels of novel ingredients, such as insect meals, microalgae, macroalgae, and yeast (*Saccharomyces cerevisiae*), have also shown to affect the fish intestinal barrier function. In Artic charr (*Salvelinus alpinus*) and gilthead seabream (*Sparus aurata*) that were fed diets with these alternative ingredients for 80 or 100 days, respectively, an increased paracellular permeability was observed <sup>[18][19]</sup>. The compromised intestine integrity enhances the risk of the intrusion of pathogens or exposure to foreign antigens from the gut lumen, thus increasing disease susceptibility and/or leading to an inflammatory reaction. Additionally, given the important role of the intestine in the immune function, the inflammatory reactions observed at the intestinal level in fish fed alternative diets may result in an impaired immunological response.

Soybean-induced enteritis has been the subject of many studies on fishmeal replacement strategies, but the other antinutritional factors that are present in plant ingredients may also affect fish health. For instance, carob seed germ meal

contains antinutritional factors, primarily tannins. Based on the morphological alterations found in the liver and distal intestine of gilthead seabream juveniles, a possible negative effect of long-term feeding with diets containing more than 34% of carob seed germ meal was suggested [20]. Lupin meal is another plant protein source that also contains antinutritional factors (phytic acid, tannins, saponins, and lectins). Barramundi (*Lates calcarifer*), when fed diets with 75% replacement of fishmeal by lupin meal (51% of dietary inclusion) for 60 days, developed liver steatosis and necrosis in the kidney, resulting in lower growth performance than in fish that were fed fishmeal-based diets [21]. Even when using plant proteins that have relatively low levels of antinutritional factors, the impacts on fish health may be found. For example, a high dietary level of wheat gluten (30% of inclusion) can have a negative impact on the intestinal and liver health of Atlantic salmon after feeding for nine weeks, with symptoms similar to gluten sensitivity in humans [22].

The technical processing steps in ingredient and diet production, such as alcohol extraction, fermentation, or heat, may inactivate or reduce the content of the antinutritional factors in plant ingredients [23]. For instance, soybean-induced enteropathy was not observed in Atlantic salmon that were fed diets with up to 25% of soy protein concentrate for 56 days [6]. Additionally, inclusion of up to 30% of bioprocessed soybean meal affected neither growth performance nor intestinal health in rainbow trout after feeding for 125 days [24]. However, highly processed plant proteins are expensive and high inclusion levels are not economically feasible in production scenarios.

Another successful strategy to counteract intestinal inflammation is the use of dietary supplements. Functional additives, comprising of a mixture of antioxidant, immunopotentiator, and trace elements, were able to partially eliminate soybean-induced enteritis in Japanese seabass [13]. The addition of probiotics (*Lactobacillus fermentum* and *Lactobacillus plantarum*) to soybean meal-based (20% of inclusion) diets did not completely prevent the development of enteritis in Atlantic salmon, but enhanced intestinal health [8]. Supplementation with butyrate (0.2% of inclusion) improved gut morphology and mitigated the symptoms of inflammation in European seabass (*Dicentrarchus labrax*), black seabream (*Acanthopagrus schlegelii*), and largemouth bass (*Micropterus salmoides*) that were fed high soybean meal ( $\pm 30\%$  inclusion) diets [25][26]. Butyrate seems to be used as a major energy source by epithelial cells of the intestine, thus stimulating cell proliferation and differentiation upon injury [26]. Dietary taurine supplementation (0.2%) also normalised the intestinal abnormalities and reduced the intestinal inflammation observed in European seabass that were fed soy protein (16.7% soybean meal and 12.8% full-fat soy) diets [25].

The search for novel ingredients to fishmeal is still in its infancy. Alternative protein sources, other than plant ingredients, may exert obvious impacts on fish health, inducing modifications at the intestinal and hepatic levels. The inclusion of more than 7.5% of whey protein concentrate in Nile tilapia (*Oreochromis niloticus*) diets resulted in swollen hepatocytes and congested hepatic blood vessels after 10 weeks of feeding [27]. This translated to disturbances in the liver function, leading to increased plasma levels of the metabolic enzymes—alanine aminotransferase (ALT) and aspartate aminotransferase (AST)—which is an indicator of liver disease. In European seabass, the inclusion of more than 16% of whey protein in diets increased the activity of liver function enzymes and disrupted kidney function after 10 weeks of feeding; although, only at 27% of dietary inclusion, histopathological signs in intestine and liver tissues were observed [28]. Insect meals are also being increasingly used as novel protein sources for fish diets. In rainbow trout, fishmeal replacement by black soldier fly prepupae meal resulted in a reduction in the absorptive epithelial surface, as well as the potential occurrence of gut inflammation [29]. In studies with juvenile barramundi, histological alterations at the intestinal epithelial level were reported after feeding totally replaced fishmeal diets (69.5% of poultry by-product meal inclusion or 63% of poultry by-product meal and 12% of insect meal) for six weeks, thus lowering the digestion and absorption surface area and impairing growth performance [30][31]. Furthermore, in these fish, increased serum levels of AST and glutamate dehydrogenase were found, associated with hepatic multifocal necrosis. Other studies also reported hepatic disturbances in fish that were fed diets containing rendered animal proteins. The inclusion of more than 24% of an animal protein blend (poultry by-product, meat and bone, spray-dried blood, and hydrolysed feather meals) induced hepatic steatosis in Japanese seabass after eight weeks of feeding [32]. In hybrid grouper, the inclusion of a blend of rendered animal proteins (poultry by-product, shrimp, and spray-dried blood meals), at levels replacing 80% of dietary fishmeal (57% inclusion level), induced hepatic steatosis by modulating the lipid metabolism-related genes and inducing hepatocyte apoptosis via the up-regulation of apoptosis-related genes [33]. Additionally, in hybrid grouper, high levels of dietary poultry meal inclusion ( $>31\%$  of the diet) induced steatosis in fish hepatocytes after eight weeks of feeding [10]. The authors related this result with the high lipid level and almost no EPA (eicosapentaenoic acid) or DHA (docosahexaenoic acid) found in poultry meal. These results serve as a reminder that the ingredients used as alternative protein sources to fishmeal are more than just protein. Hence, the processing of alternative ingredients to improve its nutritional value may be paramount, and further research is clearly necessary.

The search for sustainable alternative ingredients that promote fish health and welfare is clearly well-established as one of the priorities for aquaculture. An interesting strategy on the use of novel ingredients is its utilization for counteracting the

negative effects of some plant proteins. For instance, no hepatic damage was observed in barramundi that were fed high lupin meal diets (51% of inclusion), when 4.6% of tuna fish protein hydrolysates were included, contrary to what was observed in fish that were fed non-supplemented diets [21]. Additionally, dietary inclusion of protein hydrolysates (from krill, shrimp, or tilapia; circa 3% of inclusion) in low fishmeal (plant protein-based) diets improved gut macromorphological aspects and enhanced the apparent digestibility of dry matter and crude protein in olive flounder (*Paralichthys olivaceus*) after 11 weeks of feeding [34]. The inclusion of single cell proteins in plant protein-based diets has also provided interesting results. The inclusion of bacterial meal, of the microalgae *Chlorella vulgaris* or of the yeast *Candida utilis*, in diets with 20% of soybean meal, was highly effective in counteracting soybean-induced enteropathy in Atlantic salmon [35][36]. Oppositely, the yeast *S. cerevisiae* had no functional effects in Atlantic salmon diets [35]. However, in rainbow trout, the inclusion of a protein-rich yeast fraction (from *S. cerevisiae*) in plant protein diets, totally devoid of fishmeal, resulted in a larger fish internal intestinal surface area, which contributed to an enhanced nutrient absorption capacity [37]. Furthermore, while rainbow trout that were fed the 100% plant protein diet showed signs of moderate enteritis in the distal intestine, this inflammation was gradually alleviated with increasing incorporation of the yeast fraction in the diet (up to 15% of inclusion). As for the microalgae, the dietary inclusion of a blend of dried marine *Tisochrysis lutea* and *Tetraselmis suecica* biomass improved the gut digestive and absorptive functions of European seabass, relative to fish given a soybean meal-rich diet (35% of inclusion) for 105 days [38]. Other novel protein sources, such as insect meals, have also shown potential to ameliorate intestinal inflammation. Total replacement of fishmeal in plant protein-based diets by black soldier fly larvae meal was associated with lower enterocyte steatosis in the proximal intestine of Atlantic salmon [39]. The inclusion of defatted black soldier fly or poultry by-product meals, in diets totally devoid of fishmeal, as a replacement or a complement of plant protein-rich ingredients, resulted in improved growth and gut health in gilthead seabream and rainbow trout [40][41]. Interestingly, the combination of novel protein sources has also been shown to translate to positive impacts for fish metabolism and health. An increase in the inclusion level of full-fat black soldier fly larvae meal (35% of inclusion) in poultry by-product meal diets (totally devoid of fishmeal) resulted in no obvious hepatic lesions in barramundi [42], contrary to what has been previously observed with poultry by-product meal diets, without [30] or with 12% of full-fat black soldier fly larvae meal inclusion [31]. Thus, the dietary inclusion of alternative ingredients, such as poultry and insect meals, seems a promising strategy for counteracting the negative side effects that might be observed in non-fishmeal diets.

## 2. Impacts of Alternative Protein Sources on Fish Microbiota

Fish gut microbiota is considered a regulator of fish health [43][44], participating in key processes such as digestion, defence and immune responses, and tissue stability, among others [45][46]. One of the most relevant processes intervened by microbiota is the digestion of feed components that are indigestible for the host, leading to the synthesis of short chain fatty acids [47]. These represent a major energy source for intestinal epithelial cells and are essential for gut health. Microbial composition has been widely studied for different species and is known to vary with species, season, and life cycle events, among other factors (see the recent reviews on the subject [43][48][49]). Fish gut microbiota is also quite sensitive to dietary manipulations [50][51], and dietary modulation of its composition will change the microbiome function, so the fish will accommodate a response with physiological consequences. Most studies focusing on fish gut microbiome modulation through diet assessed the impact of functional ingredients, such as pre- or probiotics [50][52]. Indeed, alternatives to fish- and plant meals as protein sources, such as algae biomass or hydrolysates, often have functional properties when included at lower dosages [53][54] and are associated with shifts in microbial community structure.

Protein is the most important bulk nutrient, and it modulates fish gut microbiota [43][55][56]. Depending on the source, within the alternative proteins, the hydrolysates, algae, and yeasts are expected to produce alterations in the microbiota community composition. The first have short peptides that are used as substrate by several intestinal microorganisms and modulate the interactions between the microbiota and host's enterocytes [57][58]. The latter have been used as probiotics, and they are rich in compounds that can act as prebiotics, such as mannan oligosaccharides [59]. Micro- and macroalgae, however, have been highlighted as prebiotic ingredients [60][61], as well as a source of micronutrients and minerals, essential for the gut well-functioning and stability. As alternative protein sources, studies have shown controversial results, where efficiency depends greatly on inclusion rate, algae species, and biomass pre-treatments that will potentiate digestibility and nutrient utilization. Regarding algae's ability to modulate gut communities' diversity and richness (**Table 1**), results were varied and seemed to depend on fish and algae species. Feeding European seabass a diet with 15% inclusion of the cyanobacteria *Arthrospira* (formerly *Spirulina*) *platensis* biomass for 93 days reduced the feed conversion ratio (FCR) and resulted in an unfavourable growth performance [62]. No differences in gut community richness and diversity were observed; however, it was evident that there was a reduction of the abundance of several members of Proteobacteria phylum, as well as an increase of the abundance of the genera *Persicirhabdus*, *Methylobacterium*, *Acinetobacter*, and *Sediminibacterium*. In a study with Atlantic cod (*Gadus*

*morhua*) juveniles, no significant differences were found in gut microbiota or growth performance when fish were fed a diet with 10% *Ulva rigida* biomass in a 12-week trial [63]. On the other hand, in the same study, a 10% inclusion of the microalgae *Ascophyllum nodosum* reduced growth concomitantly with a reduced gut microbiota diversity and higher abundance of the genera *Psychromonas*, *Propionigenium*, and *Clostridium*. Since the first has been linked to nutritional compensation for unbalanced diets and the second is known to degrade cellulose and complex carbohydrates, as well as to produce anti-inflammatory products from its metabolism [64], the authors suggested a possible microbial modulation to compensate for any deleterious effects of the algae inclusion in the diet. When gilthead seabream juveniles were fed 10% *Tetraselmis chuii* or *Phaeodactylum tricornutum* biomass for 30 days, intestinal microbial diversity and richness were reduced, although no differences were observed in growth performance [65]. Despite those studies, others have reported positive alterations of the gut microbiota diversity when algae are used as a protein source. Feeding rainbow trout juveniles diets with 5% *Schizochytrium limacinum* biomass increased gut microbial diversity and induced a higher abundance of lactic acid bacteria (LAB) after 15 weeks [66]. An increase in microbial diversity was also found in juvenile hybrid grouper fed diets with 5 or 30% *Arthrospira platensis* biomass, associated with higher growth at the lowest inclusion level [67]. However, the most promising results have been shown when using macroalgae as alternative protein source. Feeding Senegalese sole (*Solea senegalensis*) a diet with 5% inclusion of *Ulva ohnoi* for 45 days resulted in higher microbial diversity in the anterior intestine [68]. Members of the genus *Vibrio* increased their abundance in the macroalgae-fed group; however, *Tenacibaculum*, one of the most relevant pathogens in aquaculture, had a lower abundance. A 15% dietary inclusion of *Ulva rigida* biomass increased richness in the gilthead seabream gut, but a higher inclusion (25%) had an opposite effect after 70 days [69]. Interestingly, when gilthead seabream juveniles were fed the same 25% of *Ulva rigida*, but for less time (30 days), gut bacterial communities were richer, and an increase in abundance of the genus *Lactobacillus* was observed, as well as a reduction of *Photobacterium* [70]. Indeed, the ability of macroalgae to modulate gut microorganisms has been acknowledged (see review [71]), and it assumes a special relevance in aquaculture, since fish share the environment with opportunistic microorganisms that easily become pathogenic. As in the above-mentioned study, the dietary inclusion of *Gracilaria cornea* biomass, as a protein source for 70 days, reduced the abundance of *Vibrio* and diminished *Photobacterium* genus members to a residual abundance in the gilthead seabream's intestine [69]. A reduction in the abundance of pathogenic organisms might result in the observed increased disease resistance that fish have when fed macroalgae. This was observed by some authors [70][72], who encountered lower mortalities in gilthead seabream infected with *Photobacterium damsela* subs. *piscicida*, when fed a diet with 5% *Gracilaria gracilis* biomass. Interestingly, the in vitro studies did not reveal a direct inhibitory effect against the pathogen, and this highlights that the result is probably due to the prebiotic capacities of the macroalgae; the pathogen abundance reduction is an indirect effect of the microbial network modulation. More studies are needed to address this potential disease resistance with microbiota modulation by macroalgae, linking microbiota abundance with physiological outputs.

The effects of hydrolysates as protein sources in the gut microbiota have been assessed, but not yet markedly (**Table 1**). Most studies have focused on the effects of fishmeal or plant protein replacement by hydrolysates together with other alternatives, hardening the isolation of the ingredient contributing for the observed changes. Nonetheless, the available dedicated studies could uncover some microbial modulation patterns, namely the reduction in *Vibrio* genus members abundance or dominance in gut communities. When sardine silage hydrolysate was included at 10 or 19% in European seabass larvae diets, despite the similar *Vibrio* spp. counts when compared with a commercial fish protein hydrolysate, there was a reduction of the genus members' dominant occurrences [73]. However, in some cases, the *Vibrio* sp. TYH3 strain was still found dominant in fish fed diets with sardine hydrolysate inclusion, and the authors concluded that this ingredient might promote a favourable environment for this *Vibrio* strain, which has opportunistic characteristics. Further, the authors linked this dominance occurrences with the observed higher larval resistance to *Vibrio anguillarum* challenge. Feeding juvenile turbot (*Scophthalmus maximus*) for eight weeks with diets containing up to 34% of enzymatic hydrolysates from poultry by-products resulted in a higher abundance of *Phyllobacterium*, *Sphingomonas*, and *Delftia* genera members [74]. However, this alternative protein was not well-received by fish, resulting in lower digestibility and higher FCR. A possible explanation relies on the fact that the modulated genera are frequently linked to the degradation of aromatic compounds, which might result in the accumulation of toxic metabolites in the intestinal tract, affecting gut health and digestion [74]. Nevertheless, the lowest and highest inclusion levels (8% and 34%, respectively) induced the reduction of members of genus *Vibrio* and increase of *Enterococcus*, favouring the abundance of probiotic-related microorganisms. Gilthead seabream that were fed a diet with 5% hydrolysed fish protein for 92 days presented lower gut microbial diversity, with reduced abundances of Alteromonadales and Enterobacteriales, as well as *Pseudoalteromonas* and *Vibrio* [75]. In agreement, the same species, when fed a diet with 7.5% egg white hydrolysate in a low fishmeal and fish oil diet for eight weeks, presented reduced growth performance, and this was linked with an increase in Spirochaetes and Bacteroidetes abundance, as well as a reduction in Firmicutes [76]. On the other hand, including 18% tuna hydrolysate in barramundi diets for seven weeks improved survival and intestinal histomorphology,

with a marked reduction of *Vibrio* members and increased abundance of *Psychrobacter* [77]. The latter have the capacity to secrete essential fatty acids and metabolites that will support host immune and antioxidant status [78], and this might be linked to the observed improved performance. However, data are still very limited, and only few of the published studies have used next generation sequencing (NGS) approaches. Considering the increasing awareness for reducing consumption and introducing circular economy in livestock production, the hydrolysis of waste products has gained much attention [79], and more studies are expected in the short coming future.

A similar trend is expected regarding single cell protein sources. These ingredients include bacteria, fungi (yeast), microalgae-derived products, or combinations, and they are used as protein and omega-3 sources, as well as a source of bioactive compounds (for further information on the different applications of single cell ingredients, see review [80]). From the few available studies on the effects on microbiota, it is possible to infer that those results are not always concomitant. The inclusion of 15% of baker's yeast biomass in European seabass diets for 93 days had no effect on gut microbial community richness or diversity, but growth performance was enhanced [62]. Interestingly, a lower abundance of members of the families Pseudomonadaceae, Lactobacillaceae, and Pasteurellaceae indicated that changes are directed to neither possible pathogens nor beneficial bacteria, but to a complex orchestration of abundances modulation. A lower gut microbiota diversity in gilthead seabream that were fed diets with 5% autolyzed yeast for 92 days was reported, but the abundance of members of the orders Bacillales and Clostridiales increased, whereas *Pseudoalteromonas* genus members' abundance decreased, indicating a potential enrichment of beneficial bacteria [75]. The use of yeasts, as a protein source, seems to have a limit of inclusion that is not deleterious for fish. The replacement of 20% of fishmeal with a mixture of yeasts (corresponding to 10% inclusion) in rainbow trout diets had no deleterious effect on gut microbial diversity and composition [81]. In agreement, including up to 10% torula yeast (*Cyberlindnera jadinii*) biomass to replace fishmeal in freshwater Atlantic salmon diets did not affect gut microbial diversity [82]. However, the 20% inclusion of this yeast in a plant protein-based diet reduced growth performance, in association with a reduction in microbial diversity; overall, a higher protein replacement with yeast biomass led to a reduction of LAB abundance in the gut. Further research is needed to properly address the effects of single cell protein sources in gut microbiota, as well as its inclusion limits, covering the same sources in different species, so that robust conclusions can be drawn.

**Table 1.** Summary of alternative proteins (algae, hydrolysates, and yeast-based) and their impacts on fish intestinal microbiota community.

Protein Ingredient	Animal Model and Size	Inclusion Level (%)	Trial Duration	Biological Effects		Relevant Performance, Physiological, and Feed Utilization Output	References
				Microbiota			
Algae							
<i>Ascomphyllum nodosum</i>	Atlantic cod ( <i>Gadus morhua</i> ) 123 g	10	12 weeks	↓ Diversity ↑ <i>Psychromonas</i> , <i>Propionogenium</i> , and <i>Clostridium</i> genera		↓ FBW	[63]
<i>Arthrospira</i> (formerly <i>Spirulina</i> ) <i>platensis</i>	European seabass ( <i>Dicentrarchus labrax</i> ) 19 g	15	93 days	↔ Richness and diversity ↓ Proteobacteria ↑ <i>Persicirhabdus</i> , <i>Methylobacterium</i> , <i>Acinetobacter</i> , and <i>Sediminibacterium</i> genera		↓ FBW ↑ FCR	[62]
<i>Arthrospira platensis</i>	Hybrid grouper ( <i>Epinephelus fuscoguttatus</i> × <i>E. lanceolatus</i> ) 28 g	5, 15, 30	47 days	↑ Diversity (Simpson index) in IL5 and IL30; <i>Vibrio</i> genus in IL15 ↓ <i>Tolumonas</i> genus in IL15		↔ SGR in IL15, and IL30 ↑ SGR in IL5	[67]
<i>Gracilaria cornea</i>	Gilthead seabream ( <i>Sparus aurata</i> ) 14 g	5, 15, 25	70 days	↑ Richness; <i>Pseudomonas</i> in IL15; <i>Lactobacillus</i> in IL25 ↓ <i>Vibrio</i> , loss of <i>Photobacterium</i> genus as dominant		↔ FBW	[69]
<i>Schizochytrium limacinum</i>	Rainbow trout ( <i>Oncorhynchus mykiss</i> ) 31 g	5	15 weeks	↑ Diversity, LAB		↔ FBW, K	[66]
<i>Ulva ohnoi</i>	Senegalese sole ( <i>Solea senegalensis</i> ) 11 g	5	45 days	↔ Richness ↑ Diversity in AI, <i>Vibrio</i> genus ↓ <i>Escherichia</i> and <i>Tenacibaculum</i> genera		↓ FBW, WG	[68]

Protein Ingredient	Animal Model and Size	Inclusion Level (%)	Trial Duration	Biological Effects		References
				Microbiota	Relevant Performance, Physiological, and Feed Utilization Output	
<i>Ulva pertrusa</i>	White-spotted rabbitfish ( <i>Siganus canaliculatus</i> ) 15 g	10	8 weeks	↑ <i>Ruminococcus</i> , <i>Clostridium</i> , and <i>Lachnospiraceae</i> genera	↔ FBW, SGR	[83]
<i>Ulva rigida</i>	Atlantic cod ( <i>Gadus morhua</i> ) 123 g	10	12 weeks	↔ Community composition	↔ FBW	[63]
<i>Ulva rigida</i>	Gilthead seabream ( <i>Sparus aurata</i> ) 14 g	5, 15, 25	70 days	↑ Richness in IL15; <i>Lactobacillus</i> and <i>Sphingomonas</i> in IL5 and IL15 ↓ Richness in IL25	↑ FBW in IL25	[69]
<i>Ulva rigida</i>	Gilthead seabream ( <i>Sparus aurata</i> ) 14 g	25	30 days	↑ Richness, <i>Lactobacillus brueckii</i> ↓ <i>Photobacterium</i> genus	↑ SR during challenge with <i>Photobacterium damselae</i> subs. <i>piscicida</i>	[70]
<i>Tetraselmis chuii</i> or <i>Phaeodactylum tricornutum</i>	Gilthead seabream ( <i>Sparus aurata</i> ) 50 g	10 alone 10 w/ <i>Bacillus</i>	30 days	↓ Richness and diversity	↑ Intestinal epithelial damage	[65]
Hydrolysates						
Fish protein	Gilthead seabream ( <i>Sparus aurata</i> ) 122 g	5	92 days	↔ Richness ↓ Diversity (PD whole tree), Alteromonadales and Enterobacteriales, <i>Pseudoalteromonas</i> and <i>Vibrio</i> genera	↔ FBW, SGR, FER	[75]
Sardine	European seabass ( <i>Dicentrarchus labrax</i> ) larvae 8 DPH	10, 19	25 days	Changes in <i>Vibrio</i> abundance (limited, due to method)	↓ FBW ↑ SR, when challenged with <i>V. anguillarum</i>	[73]
Tuna	Barramundi ( <i>Lates calcarifer</i> ) 2.5 g	18	7 weeks	↓ <i>Vibrio</i> ↑ <i>Psychrobacter</i>	↑ SR, histomorphology	[77]
Egg white	Gilthead seabream ( <i>Sparus aurata</i> ) 20 g	7.5	8 weeks	↔ Diversity ↑ Spirochaetes, Bacteroidetes ↓ Richness, Firmicutes	↓ FBW, K, FI, SGR	[76]
Feather meal	Atlantic salmon ( <i>Salmo salar</i> ) 305 g	20	12 weeks	↔ Dominant bacteria ↑ Abundance of allochthonous bacteria (families Corynebacteriaceae, Lactobacillaceae and Streptococcaceae), Pseudomonadaceae in PI ↓ Vibrionaceae in PI	↔ FBW, SGR	[84]

The utilization of insect meal for fishmeal and plant protein replacement in aquaculture has been recently addressed by many researchers, and several studies on the effects of this protein source on fish gut microbiota are available. Among the studied insects, the black soldier fly is undoubtedly the most studied (Table 2), followed by the yellow mealworm. These ingredients are highly available for a broader usage, and studies have shown an overall improvement in gut health and associated microbiota. Insects are generally known to improve feed intake, considering the results of most studies, this might be due to the effect of microbiota. Within the insect's composition, chitin is the most relevant and abundant compound. This mucopolysaccharide polymer is composed of β-1,4-linked N-acetylglucosamine units; since it is not degraded by fish, it is considered an insoluble fibre with potential prebiotic properties [86][87]. Additionally, chitin and its deacetylate derivate, chitosan, are known for having antimicrobial effects on several Gram-negative bacteria [87], and this might be the preferred mode of action for the exerted effects. Although some authors have reported no effect of insect meal on gut microbiota composition diversity or richness [62][98][99][100], the majority indicates an increase of diversity [77][91][92][93][94][96][97][98][99][100]. The increase in LAB abundance is one of the most common features that insect meal produces in fish gut microbiota [92][93][94][96][97][98][99][100]. Nevertheless, feeding a diet with 10% hydrolysed yellow mealworm or superworm (*Zophobas morio*) meals reduced *Lactobacillus* and *Carnobacterium* abundance, although no differences in growth were observed. These and other LAB are, in general, regarded as beneficial bacteria that inhibit the growth of pathogenic microorganisms, through the secretion of inhibitory substances, such as bacteriocins and hydrogen peroxide, among others, acting as immunostimulant and improving host's health, in addition to playing a relevant role in degrading indigestible carbohydrates, such as fibre and starch (see review [51]). In addition to LAB, others are modulated by insect meal and linked with fish health. In Atlantic salmon that were fed a diet with 15% black soldier fly meal for 16 weeks, an increase in the abundance of *Brevinema andersonii* and Spirochaetaceae members was found, and the authors linked those changes with the expression of genes related to immune response and gut barrier function [91]. In rainbow trout, the dietary inclusion of black soldier fly prepupae meal (up to 20%) increased the abundance of *Mycoplasma*, a microorganism that produces lactic and acetic acids as major metabolites, with beneficial consequences for the gut mucosa [95]. Additionally, an increase in *Psychrobacter* abundance was reported in barramundi that were fed a diet with 18% inclusion of black soldier fly meal for seven weeks [77]. The members of this genus produce (and make available) essential fatty acids and metabolites that enhance the host's immune and antioxidant status [64].

**Table 2.** Summary of insects' meal and their impacts on fish intestinal microbiota community.

↔ Without differing from control; ↓ significantly lower than the control; ↑ significantly higher than the control; AI = anterior intestine; FBW = final body weight; FCR = feed conversion ratio; FER = feed efficiency; FI = feed intake; IL = inclusion level; K = condition factor; LAB = lactic acid bacteria; PI = posterior intestine; PP = plant protein-based diets; SGRs = specific growth rate; SR = survival rate; WG = weight gain.

Protein Ingredient	Animal Model and Size	Inclusion Level (%)	Trial Duration	Microbiota	Relevant Performance, Physiological, and Feed Utilization Output	Reference
Insects' Meal						
Black soldier fly ( <i>Hermetia illucens</i> )	Atlantic salmon ( <i>Salmo salar</i> ) 49 g	60	8 weeks	↑ Diversity in mucosa-associated community, Bacillaceae family, <i>Bacillus</i> , <i>Actinomyces</i> ↓ Digesta-associated community	↔ SGR	[91]
Black soldier fly ( <i>Hermetia illucens</i> )	Atlantic salmon ( <i>Salmo salar</i> ) 1400 g	15	16 weeks	↑ Diversity and richness, <i>Brevinema andersonii</i> , Spirichaeetaceae	↔ SGR	[91]
Black soldier fly ( <i>Hermetia illucens</i> )	Barramundi ( <i>Lates calcarifer</i> ) 3 g	18	7 weeks	↑ Diversity (Shannon and Simpson index), <i>Psychrobacter</i> ↓ <i>Vibrio</i>	↔ FBW, SGR	[77]
Black soldier fly ( <i>Hermetia illucens</i> )	European seabass ( <i>Dicentrarchus labrax</i> ) 19 g	15	93 days	↔ Diversity and richness ↓ Proteobacteria and Bacteroidetes, Weeksellaceae, and Prevotellaceae ↑ <i>Paracoccus</i>	↑ FBW ↓ FCR	[62]
Black soldier fly ( <i>Hermetia illucens</i> )	European seabass ( <i>Dicentrarchus labrax</i> ) 6 g	30	12 weeks	↔ Diversity and richness ↑ <i>Anaerococcus</i> , <i>Cutibacterium</i> , <i>Pseudomonas</i> , and Firmicutes:Bacteroidetes ratio	↔ FBW, FCR, K	[100]
Black soldier fly ( <i>Hermetia illucens</i> )	Gilthead seabream ( <i>Sparus aurata</i> ) 30 g	30	12 weeks	↔ Diversity and richness ↑ <i>Staphylococcus</i> , <i>Hafnia</i> , and <i>Aeromonas</i>	↔ FBW, FCR, K	[100]
Black soldier fly ( <i>Hermetia illucens</i> )	Siberian sturgeon ( <i>Acipenser baerii</i> ) 640 g	15	60 days	↑ <i>Bacillus</i> , <i>Lactobacillus</i> , and <i>Enterococcus</i> Overall positive and strong modulation	↔ FBW, SGR, FCR ↓ Mucosa thickness	[92]
Black soldier fly ( <i>Hermetia illucens</i> )	Meagre ( <i>Argyrosomus regius</i> ) 18 g	10, 20, 30	9 weeks	↔ Diversity and community composition	↑ Gut histomorphology alterations	[90]
Black soldier fly ( <i>Hermetia illucens</i> )	Rainbow trout ( <i>Oncorhynchus mykiss</i> ) 178 g	20, 40	78 days	↑ Diversity, structure, and composition (mainly in IL20) ↑ Digesta associated LAB	↔ WG, FCR	[98]
Black soldier fly ( <i>Hermetia illucens</i> )	Rainbow trout ( <i>Oncorhynchus mykiss</i> ) 200 g	30	5 weeks	↑ Diversity (Shannon index), richness (Chao index), LAB, <i>Corynebacterium</i> , Bacillaceae ↓ Proteobacteria:Firmicutes ratio	↔ WG, FI	[93]
Black soldier fly ( <i>Hermetia illucens</i> )	Rainbow trout ( <i>Oncorhynchus mykiss</i> ) 53 g	20	71 days	↑ LAB (mainly <i>Lactobacillus</i> and <i>Enterococcus</i> )	↔ SGR, FCR, villus height, mucosa thickness	[97]
Black soldier fly ( <i>Hermetia illucens</i> )	Rainbow trout ( <i>Oncorhynchus mykiss</i> ) 66 g	10, 20, 30	12 weeks	↔ Richness ↑ Diversity (Shannon and Simpson) in IL20, <i>Mycoplasma</i> ↓ <i>Aeromonas</i> and <i>Citrobacter</i>	↔ FBW, SGR	[95]
Black soldier fly ( <i>Hermetia illucens</i> )	Rainbow trout ( <i>Oncorhynchus mykiss</i> ) 66 g	10, 20, 30	12 weeks	↑ Diversity (Shannon and Simpson), richness (Chao1), Firmicutes and Actinobacteria, LAB (mainly Leuconostocaceae and Lactobacillaceae), and Actinobacteria ( <i>Actinomyces</i> , <i>Corynebacterium</i> )	↔ FBW, SGR, FCR	[94]

Biological Effects						
Protein Ingredient	Animal Model and Size	Inclusion Level (%)	Trial Duration	Microbiota	Relevant Performance, Physiological, and Feed Utilization Output	References
Black soldier fly ( <i>Hermetia illucens</i> )	Rainbow trout ( <i>Oncorhynchus mykiss</i> ) 54 g	8, 23, 45 in PP-based diet	12 weeks	↔ Firmicutes:Proteobacteria ratio ↑ Diversity and richness than PP diet (restored) in IL23 and IL45, <i>Actinomyces</i> , <i>Bacillus</i> , <i>Dorea</i> , <i>Enterococcus</i> , <i>Mycoplasma</i>	↑ Growth performance, mainly in IL8 combined with poultry meal ↔ Gut barrier integrity	[99]
Black soldier fly ( <i>Hermetia illucens</i> )	Rainbow trout ( <i>Oncorhynchus mykiss</i> ) 100 g	15	131 days	↑ Richness, Firmicutes (mainly <i>Bacillus</i> ), <i>Lactobacillus</i> , and <i>Bacillus</i> ↓ Proteobacteria, <i>Aeromonas</i> genus	↔ FBW, SGR	[96]
Housefly ( <i>Musca domestica</i> )	European seabass ( <i>Dicentrarchus labrax</i> ) 6 g	30	12 weeks	↔ Diversity and richness ↑ <i>Anaerococcus</i> , <i>Cutibacterium</i> , and <i>Pseudomonas</i>	↔ FBW, FCR, K	[100]
Housefly ( <i>Musca domestica</i> )	Gilthead seabream ( <i>Sparus aurata</i> ) 30 g	30	12 weeks	↔ Diversity ↓ Richness ↑ Firmicutes:Bacteroidetes ratio	↔ FBW, FCR, K	[100]
Indian house cricket ( <i>Gryllodes sigillatus</i> )	Rainbow trout ( <i>Oncorhynchus mykiss</i> ) 53 g	20	71 days	↑ LAB (mainly <i>Lactobacillus</i> and <i>Enterococcus</i> )	↑ FCR ↓ SGR, villus height, mucosa thickness	[97]
Yellow mealworm ( <i>Tenebrio molitor</i> )	European seabass ( <i>Dicentrarchus labrax</i> ) 5 g	50	70 days	↔ Diversity	↔ FBW	[89]
Yellow mealworm ( <i>Tenebrio molitor</i> )	European seabass ( <i>Dicentrarchus labrax</i> ) 6 g	30	12 weeks	↔ Diversity and richness ↑ <i>Anaerococcus</i> , <i>Cutibacterium</i> , <i>Pseudomonas</i> , and Firmicutes:Bacteroidetes ratio	↔ FBW, FCR, K	[100]
Yellow mealworm ( <i>Tenebrio molitor</i> )	Gilthead seabream ( <i>Sparus aurata</i> ) 105 g	50	163 days	↑ Proteobacteria:Firmicutes ratio ↓ Firmicutes:Bacteroidetes ratio	↔ FBW	[89]
Yellow mealworm ( <i>Tenebrio molitor</i> )	Gilthead seabream ( <i>Sparus aurata</i> ) 30 g	30	12 weeks	↔ Diversity and richness ↑ <i>Staphylococcus</i> , <i>Hafnia</i> , and <i>Aeromonas</i>	↔ FBW, FCR ↑ K	[100]
Yellow mealworm ( <i>Tenebrio molitor</i> )	Rainbow trout ( <i>Oncorhynchus mykiss</i> ) 115 g	50	90 days	↑ Diversity (Simpson index)	↔ FBW	[89]
Yellow mealworm ( <i>Tenebrio molitor</i> )	Rainbow trout ( <i>Oncorhynchus mykiss</i> ) 53 g	20	71 days	↑ LAB (mainly <i>Lactobacillus</i> and <i>Enterococcus</i> )	↔ SGR, FCR, mucosa thickness ↓ Villus height	[97]

Researchers have also indicated that microbiota modulation is dependent not only on fish species, but also on the insect species used as ingredient. The effect of 50% inclusion of yellow mealworm in gilthead seabream, European seabass, and rainbow trout was compared, and differential effects were reported [89]. Microbial diversity remained unchanged in gilthead seabream and European seabass, whereas, in rainbow trout, the Simpson's diversity index was higher. Moreover, in gilthead seabream, the ratio Proteobacteria:Firmicutes increased, whereas the Firmicutes:Bacteroidetes reduced, and the same was not observed for the other species, indicating highly variable modulation depending on fish species. However, in this study, fish had different sizes (105, 5, and 115 g for seabream, seabass, and trout, respectively), the feeding trials had different durations (163, 70, and 90 days, respectively), and these differences might harden the comparisons. In another study, the effects of the yellow mealworm, black soldier fly, and housefly (*Musca domestica*) meals, included at 30%, were compared in gilthead seabream and European seabass diets for three months, and different effects on microbiota were also found, depending on fish and insect species [100]. In European seabass, insect meals (regardless the species) increased the abundance of *Anaerococcus*, *Cutibacterium*, and *Pseudomonas*, whereas, in gilthead seabream, the increase in *Staphylococcus*, *Hafnia*, and *Aeromonas*, as well as the reduction of members of Firmicutes phylum, was noticeable. On the other hand, the Firmicutes:Bacteroidetes ratio increased in European seabass that were fed with yellow mealworm and black soldier fly meals, and the same was true when gilthead seabream were fed a diet with housefly meal [100]. In rainbow trout, the effects of four different insect meals (the black soldier fly, yellow mealworm, Indian house cricket (*Gryllodes sigillatus*), and Turkestan cockroach (*Blatta lateralis*)), at 20% dietary inclusion, were tested for 71 days [97]. The authors found that yellow mealworm and cockroach meals improved growth,

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whereas black soldier fly meal had no effect, and the cricket meal reduced the performance. Although there was higher abundance of LAB (mainly *Lactobacillus* and *Enterococcus*) in all groups, in the cockroach meal-fed group, an increase in *Clostridia coccoides* abundance was observed. This microorganism is linked with the maintenance of gut function, and it might be a reason for the higher villus height observed in this group, which is a positive indication. One more factor that sums to the complexity in assessing the effects of insect meals in gut microbiota is the insect life cycle stage. Although most studies were performed with insect larvae as a biomass source, different insect stages and processing will affect gut microbial modulation. In rainbow trout juveniles, the effects of black soldier fly meal, which was defatted larvae or prepupae, were compared for five weeks, at a 30% dietary inclusion level [93]. Insect meal increased microbial diversity, richness, and LAB's abundance, a higher abundance of *Corynebacterium* genus and *Bacillaceae* was found in the groups that were fed with insect larvae, but not with prepupae. Notably, the increase in *Corynebacterium* members—a genus known for producing lipases—occurs in groups fed with diets with high lipidic content, supporting a strong correlation between microbiota and dietary nutritional sources.

From the above-mentioned studies, the overall positive effect of insect meal on fish gut microbiota and health is evident; however, further studies should focus on unravelling the relationships between microbial modulation and physiological outputs, as well as responses and survival when facing insults, such as stress or an infection.

### 3. Impacts of Alternative Protein Sources on Immune Function and Disease Resistance

Without differing from control; ↓ significantly lower than the control; ↑ significantly higher than the control; FBW = final body weight; FCR = feed conversion ratio; FI = feed intake; IL = inclusion level; K = condition factor; LAB = lactic acid bacteria; PFI = plant protein inclusion; SGR = specific growth rate; WFI = weight gain. The competition between the nutrients available for maintenance purposes, for the functioning of the immune system, and for body protein deposition in growing animals. This takes particular importance for the aquaculture industry, where nutrition can have significant health implications for fish. Therefore, best practices on diet formulation are of major importance, as feeds represent the leading expenditure to the aquaculture industry [102]. However, keeping in mind the most recent trends in feed formulation for the use of alternative protein sources to decrease the dependency of fishmeal, it is fundamental to evaluate the implications of this new paradigm for fish immune function and disease resistance.

Plant protein ingredients are among the alternatives to fishmeal most studied in the last couple of decades, as a strategy to contribute to aquaculture sustainability and improve cost-effective production. However, most plant protein ingredients have a wide variety of antinutritional factors, which may interfere with fish performance and health, including alterations on immunity, inflammation process, and lower resistance to diseases. Fishmeal replacement, by a blend of plant proteins (i.e., wheat gluten, soybean meal, and soy protein concentrate), negatively affected the immune status of the turbot juveniles fed the highest inclusion levels of plant proteins (>50% of inclusion) [103]. Similarly, fishmeal replacement, by a mixture of plant proteins (i.e., soybean meal, wheat meal, wheat gluten, and corn gluten), in the diets of European seabass decreased plasma immunoglobulins, blood monocytes, and gut interleukin-10 (IL-10) mRNA expression [104]. A five-months feeding trial with low dietary fishmeal levels in European seabass also led to detrimental immune responses [105]. For instance, an up-regulation of IL-1 $\beta$ , tumour necrosis factor  $\alpha$  (TNF $\alpha$ ), major histocompatibility complex-II (MHCII), and cyclooxygenase-2 (COX-2) was observed, whereas the gut-associated lymphoid tissue decreased its capacity to respond to a sub-lethal dose of *Vibrio anguillarum* via anal inoculation. Gilthead seabream that were fed a diet with total fishmeal replacement, by a blend of wheat gluten, broad bean, soybean, pea, and sunflower meals, showed lower expression in the genes related to pro-inflammatory response, such as IL-1 $\beta$ , IL-6, and COX-2, as well as in immune-related genes, such as immunoglobulin (Ig) M, in line with the high mortality rates observed [106]. Likewise, negative health-related impacts in the gut of turbot were reported after feeding high levels of corn gluten meal, inducing enteritis, and decreasing intestinal immunity, by increasing the inflammatory cytokine transcripts IL-1 $\beta$ , IL-8, and TNF- $\alpha$  [107]. High levels of soybean meal inclusion in diets for several fish species have also been documented to trigger intestinal inflammation [108][109][110]. These adverse effects of plant protein inclusion are known to be related to the presence of antinutritional factors. Although many alternatives have been tested to remove antinutrients and enhance plant protein utilization, few studies focused on immune responses. For instance, dietary inclusion of a fermented plant protein concentrate, up to 40%, in diets for juvenile olive flounder did not affect lysozyme activity nor survival after eight weeks of feeding [111]. In another study, *Aspergillus oryzae*-fermented rapeseed meal was included in diets for red seabream (*Pagrus major*), in order to replace 50% of fishmeal. An increase in lysozyme, respiratory burst, and bactericidal activities in red seabream fed fermented rapeseed meal for 56 days was observed, suggesting a better immune response in these fish, compared to the ones fed with non-fermented rapeseed meal [112]. In contrast, hybrid grouper fed soybean meal or fermented soybean meal for 10 days developed enteritis and showed an increase in the expression of pro-inflammatory genes (IL-1 $\beta$ , IL-8, IL-17, and TNF $\alpha$ ), as well as a decrease in anti-inflammatory genes (IL-4, IL-10, and transforming growth factor  $\beta$ 1—TGF $\beta$ 1) and immune parameters, such as lysozyme, complement factors C3 and C4, and IgM [110].

Rendered animal by-products, such as blood meal, poultry by-product meal, feather meal, and bone meal, have also been targeted as fishmeal substitutes, due to their nutrient profile and digestibility. Spray-dried blood and plasma proteins have been recognised as high quality feed ingredients for farmed animals and have been reported to enhance innate immunity in fish. In fact, gilthead seabream that were fed diets with 3 and 6% spray-dried plasma from porcine blood for 60 days showed improved innate immune responses by enhancing serum lysozyme and bactericidal activities <sup>[113]</sup>. Porcine soluble, originating from porcine mucosal tissues, is a by-product of heparin production that has also been tested in diets for fish. In particular, juvenile rice field eel (*Monopterus albus*) were fed spray-dried porcine soluble at 2.5 and 5% dietary inclusion levels for 56 days, which translated to positive effects at the highest dietary level by enhancing serum complement factors (C3 and C4) and lysozyme activity, while improving gut health <sup>[114]</sup>. Poultry by-product meal is also a cost-effective ingredient to replace fishmeal. Cobia (*Rachycentron canadum*) juveniles that were fed diets with up to 60% of poultry by-product meal for 10 weeks did not show any changes in haematocrit, haemoglobin, red blood cell, and total immunoglobulins, compared to fish fed a fishmeal-based diet <sup>[115]</sup>. In contrast, the replacement of fishmeal by chicken plasma powder in diets for largemouth bass negatively affected innate immunity. While inclusion levels of chicken plasma powder at 5 and 10% did not change lysozyme, classical complement pathway, and respiratory burst activities after 12 weeks of feeding, compared to control specimens, a drop in those immune parameters was observed in largemouth bass that were fed diets with 15% of chicken plasma powder <sup>[116]</sup>. Similarly, total substitution of fishmeal, by poultry by-product meal, negatively influenced the liver health, histological traits of different organs, and immune response in juvenile barramundi after six weeks of feeding <sup>[30]</sup>. An innovative study tested the effects of a blend of poultry by-product, shrimp, and spray-dried blood meals in diets of juvenile hybrid grouper for eight weeks. At the end of the feeding trial, high levels of dietary processed animal proteins induced hepatic steatosis in hybrid grouper by modulating lipid metabolism-related genes, hepatocyte apoptosis via the up-regulation of apoptosis-related genes, and triggered an inflammatory response via up-regulation of inflammatory cytokines, thus suggesting that hybrid grouper immunity could be impaired, to some extent, by feeding high levels of a blend of processed animal proteins <sup>[33]</sup>. A recent study assessed the effects of partial replacement of plant proteins by poultry by-product meal (30 and 60% substitution), or in combination with black soldier fly meal, on the inflammatory and immune biomarkers of rainbow trout juveniles fed for 91 days. At the end of the feeding trial, IL-1 $\beta$ , IL-10, TGF $\beta$ , COX-2, and T-cell receptor  $\beta$  (TCR $\beta$ ) gene expression levels in both intestine and head kidney suggested there were no signs of inflammation, and the study concluded that both insect and poultry by-product meals appear to be valid protein sources in fishmeal-free diets <sup>[99]</sup>. The latter approach of combining poultry by-product and black soldier fly meals was also assessed in barramundi juveniles fed for six weeks. While total fishmeal replacement, by both protein sources, induced detrimental effects at the end of the feeding trial, diets with 55% of fishmeal replacement, by a blend of poultry by-product and insect meals (i.e., 31 + 12% of inclusion, respectively), allowed for enhanced survival against *Vibrio harveyi* infection, in line with an increase in serum lysozyme activity and relative expression of complement factors C3 and C4 in the head kidney <sup>[31]</sup>.

Insects have more recently been considered an appropriate protein source for fish diets, and research efforts are also being directed to study host immune responses, since insect chitin could contribute to boost innate immunity. A recent study with rainbow trout juveniles reported positive effects of black soldier fly larvae inclusion at 8 and 16% in diets with high levels of soybean meal. In this study, insect meal was effective in preventing enteritis by lowering the expression of prostaglandin and interferon regulatory factor 1 in the gut, while the highest inclusion level also improved serum lysozyme activity <sup>[7]</sup>. In pre-smolt Atlantic salmon, the dietary inclusion of black soldier fly larvae meal and paste were assessed as substitutes of fishmeal and plant proteins during a seven-week feeding trial <sup>[117]</sup>. While a moderate inclusion of black soldier fly larvae meal (i.e., 12.5%) augmented plasma lysozyme levels and tended to improve the phagocytic activity in head kidney cells, the highest inclusion level (i.e., 25% of insect meal) increased interferon  $\gamma$  and reduced IgM in the distal intestine. In contrast, an inclusion of 35% of black soldier fly larvae paste improved the distal intestine histology. This study also showed minor effects on the expression profile of proteins in skin mucus and no effects on immune markers in splenocytes by the dietary inclusion of black soldier fly larvae meal and paste. Additionally, in Atlantic salmon, another study assessed the effects of total or partial (66%) fishmeal replacement, by black soldier fly larvae meal, on isolated head kidney leukocytes, after simulating an exposure to bacterial or viral antigens. While inflammatory-related gene expression in head kidney cells was not affected by dietary treatments, Atlantic salmon fed with insect meal presented a decrease in the expression of LOX5, a gene coding for an enzyme responsible for the biosynthesis of inflammatory mediators <sup>[118]</sup>. A study performed with hybrid tilapia (Nile  $\times$  Mozambique; *Oreochromis niloticus*  $\times$  *O. mozambicus*) approached the suitability of dietary inclusion of frass, a by-product of the black soldier fly larval meal industry that includes larval excrement, exoskeleton sheds, and residual feed ingredients, along with abundant nutrients, chitin, and beneficial microbes <sup>[119]</sup>. In this study, frass was included (up to 30%) as partial replacement of a soybean meal, wheat short, and corn meal blend; at the end of the feeding trial (i.e., 12 weeks), the serum complement activity increased significantly in the hybrid tilapia fed the highest frass inclusion level (30%). Moreover, dose-dependent trends in survival, against both *Flavobacterium columnare* and *Streptococcus iniae* challenges, were observed, suggesting that dietary frass

from black soldier fly larvae could be beneficial by improving innate immunity and disease resistance of hybrid tilapia against bacterial infection. In an innovative study, black soldier fly larvae were injected with bacteria to boost insect immunity and then incorporated in diets for rainbow trout [120]. Dietary treatments included fishmeal replacement by 25 or 50% of non-immunised or immunised black soldier fly larvae meals. While serum lysozyme levels were increased in rainbow trout that were fed both levels of immunised black soldier fly meal, compared to controls and those fed non-immunised insects, extracts from the immunised black soldier larvae showed inhibitory activity against fish pathogenic bacteria.

Recent research also focused on the potential role of the yellow mealworm, given its excellent nutritional and commercial values. For instance, European seabass that were fed a diet with 25% yellow mealworm inclusion for six weeks showed a drop in serum myeloperoxidase and nitric oxide levels, while lysozyme activity and trypsin inhibition augmented significantly [121]. A similar approach in juvenile yellow catfish (*Pelteobagrus fulvidraco*) assessed the effects of partial fishmeal replacement by yellow mealworm (up to 75%) at the end of a five-week feeding trial, as well as 24 h following bacterial (*Edwardsiella ictaluri*) challenge [122]. While plasma IgM levels were augmented in a dose–response manner with the increasing dietary yellow mealworm content at the end of feeding trial, plasma lysozyme activity was enhanced in yellow catfish that were fed yellow mealworm diets at 24 h following bacterial challenge, in line with the observed up-regulation of immune-related genes (e.g., MHCII, IL-1 $\beta$ , and IgM) in these groups. This study also reported an improved survival against *Edwardsiella ictaluri* in fish that were fed the highest yellow mealworm inclusion level, suggesting a great potential of this insect meal as a strategy to improve yellow catfish immune response and disease resistance. A recent study with juvenile olive flounder was designed to replace 20 to 80% of fishmeal by yellow mealworm (13 to 52% of inclusion), as it was a locally available and affordable protein ingredient, during an eight-week feeding trial [123]. Although this study reported an immunostimulatory effect, to some degree, in olive flounder that were fed the highest yellow mealworm inclusion level, the authors suggested lower inclusion levels (i.e., 40% of fishmeal replacement) to avoid side effects in terms of fillet quality.

Beyond using yellow mealworm and black soldier fly meals as novel protein sources, other studies approached the suitability of other insect species in aquafeeds. Low dietary levels of housefly pupae enhanced peritoneal leukocyte phagocytic activity (i.e., 10 days of feeding at 0.75 and 7.5% inclusion levels) and disease resistance of the red seabream against *Edwardsiella tarda* (i.e., 2 months of feeding at 5% inclusion level) [124]. In contrast, housefly maggot meal, as a fishmeal substitute, in diets for juvenile barramundi decreased plasma lysozyme activity when included at 30% during an eight-week feeding trial [125]. Additionally, the effects of diets containing superworm larvae meal (i.e., 15% and 30%) on Nile tilapia innate immunity were studied after 12 weeks of feeding and following challenge with lipopolysaccharide (LPS) [126]. This study showed an increased lysozyme activity in the serum of Nile tilapia that were fed superworm larvae meal, whereas neutrophils and alternative complement activity in serum were also augmented in those groups after LPS-challenge. Indeed, the potential of using insect meal as a fishmeal substitute, while improving immune machinery and disease resistance, is high; further studies should be directed to develop fortified and sustainable aquafeeds with high inclusion levels of insect meals. Another study approached the possibility of including insect meal, originated from *Spodoptera littoralis*, in diets for Nile tilapia juveniles. Since immune parameters were not altered by increasing the level of this insect meal, the study suggested that fishmeal replacement by this novel ingredient (up to 20%) is feasible for Nile tilapia diets [127]. Other novel insect meal sources were also tested in mirror carp (*Cyprinus carpio* var. *specularis*) diets, during an 11-week feeding trial [128]. In this study, silkworm pupae (*Bombyx mori*) and ragworm (*Nereis virens*) meals were used to replace fishmeal at 24–28%. While no changes were observed for haematological parameters or plasma lysozyme activity, the alternative complement pathway was enhanced in mirror carp fed diets with silkworm pupae inclusion, which could be linked to the presence of long-chain polysaccharides in the pupae meal.

Macro- and microalgae have been introduced as an added-value dietary novel commodity in the last years, thus providing essential and bioactive nutrients to farmed fish. Several species have been addressed as natural sources for improving innate immunity, such as *Ulva rigida*, *Gracilaria gracilis*, and *Arthrospira platensis*, among others. For instance, Nile tilapia juveniles were fed diets with 25% inclusion of *Ulva rigida*, *Crassiphycus corneus* (formerly *Hydropuntia cornea*), and *Scenedesmus almeriensis* for 30 days, being that the diet with *Scenedesmus almeriensis* inclusion was able to enhance respiratory burst, alternative complement pathway and lysozyme activities [129]. An increase in the alternative complement pathway was also observed in gilthead seabream juveniles fed a diet with 25% inclusion of *Ulva rigida* for 30 days, which translated to higher resistance against *Photobacterium damsela* subsp. *piscicida* challenge [70]. An improved disease resistance against the same bacterial pathogen was also observed in gilthead seabream juveniles that were fed *Gracilaria gracilis* powder at 5% dietary inclusion after 52 days, a fact that contrasted with the lower plasma lysozyme activity observed in those specimens before disease challenge, compared to their counterparts that were fed the control diet [72]. In a similar study, European seabass were fed diets with 2.5 and 5% inclusion of *Gracilaria gracilis* powder for 47 days, but no significant changes were observed in innate immune parameters, compared to specimens that were fed the

control diet <sup>[130]</sup>. Similarly, Atlantic salmon that were fed diets at 5, 10, and 15% inclusion levels of dillisk (*Palmaria palmate*) for 14 weeks did not show any changes in haematological parameters, or in plasma lysozyme and alternative complement pathway activities <sup>[131]</sup>.

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