Chapter 5

The Potential Impacts of Soy Protein on Fish Gut Health

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Additional information is available at the end of the chapter

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Abstract

Soy protein is the major source of protein as fishmeal replacement in fish feed because of its worldwide availability and low price. However, the presence of high carbohydrate content along with saponins, lectins, and phytates can have a negative impact on fish gut health. Based on the literature and our lab studies, dietary soybean meal can cause a dose-dependent type of distal intestine inflammation called enteritis in commercial fish species including salmonids. This leads to reduced absorptive capacity, increased mucus secretion, hyperpermeability, and leucocyte infiltration in the lamina propria and submucosa, also inducing the pro-inflammatory cytokine genes expression, including Il-1β, Il-8, and Tnf-α. In addition, dietary soy may alter the composition and population of the gut microbiota via providing nutrients and energy that preferentially support the growth of some gut bacteria. This chapter summarizes the current knowledge of the effects of soy protein on the enteritis and gut microbiota.

Keywords: aquaculture, fish feed, soy protein, growth performance, enteritis, microbiome

1. Introduction

Soybean meal (SBM) is one of the most commonly used alternative plant-based ingredients to replace marine derived fishmeal (FM) in aquafeed. Relatively, high protein content and favorable amino acid profile of SBM approaches the nutritional requirement of many cultured species [1–4]. In carnivorous fish species almost 20–40% fishmeal protein can be replaced by SBM protein without compromising growth, feed utilization performances, and

gut health [5]. It is also well documented that high dietary soy protein inclusion resulted in lower feed intake, reduced weight gain, morphological changes of distal intestinal epithelium, and abnormal health condition of fish [6–12]. The challenges behind the high inclusion of soy protein in aquafeed includes the limiting amino acids methionine, presence of high carbohydrate level which negatively influences mainly carnivorous species, and the presence of different antinutritional factors (ANFs) [1–3]. To overcome the challenges, several techniques have been attempted viz. using different processing techniques (heat and enzymatic treatment, bioprocessing, fermentation, etc.) to improve the soy ingredient profiles like increased protein levels, decreased levels of ANFs, and enhanced digestibility [13–15]. For balancing amino acids profile, a balance mixture of soy protein with other plant ingredients protein and crystalline amino acids supplementation were also practiced. However, SBM of standard quality is used in carnivorous fish diets only at relatively low levels due to its negative effects on gut health in several fish species [16]. Different soy protein sources have been found to modulate many aspects of gastrointestinal tract (GIT) health within fish species, including the histological composition, immune status, and the overall intestinal microbiota [17–23]. Intestinal morphology, gut-associated immunity, and microbial community are closely interacting with each other. The present chapter addresses the potential impacts of different soy protein inclusion in aquafeed on gut health condition of fish with special emphasis on gut morphology, soybean meal-induced enteritis (SBMIE), gut-associated immunity, and gut microbiota.

2. Effect of soybean meal inclusion in aquafeed to induced enteritis (SBMIE) in fish

When studying enteritis in fish, it is important to consider all cell types involved in a correct function of the gastrointestinal tract (GIT). Like mammals, fish gut is critical for nutrient digestion and absorption, immunity, and interaction with the environment [24–26]. In fish, a simpler division of the GIT is described as compared to mammals: two different segments of the gut are distinguished: proximal or anterior intestine and distal or posterior intestine (**Figure 1a,b**). Of these, distal intestine is where most of the nutrient absorption occurs and is the object of study of SBMIE in fish [28–33].

The word enteritis refers to an inflammatory process happening in the gut, which can be caused by a diverse range of factors. The symptoms that define the condition are a shortening of the mucosal folds, a loss of the normal supranuclear vacuolization of the absorptive cells in the intestinal epithelium, a widening of the central stroma within the mucosal folding, with increased amounts of connective tissue, a profound infiltration of inflammatory cells in the lamina propria [4, 6, 19, 34–37], an increased presence of IgM [38], an increased amount of goblet cells in the epithelium, as well as a decreased height of the microvilli together with increased microvillar vesicle formation [36]. Baeverfjord and Krogdahl [6] also described this condition as "a non-infectious sub-acute inflammation of the distal intestine." Typically, FM replacement by SBM in fish diets is between 20 and 40% at which signs of enteritis are detected in a large variety of both marine and freshwater species including omnivores and

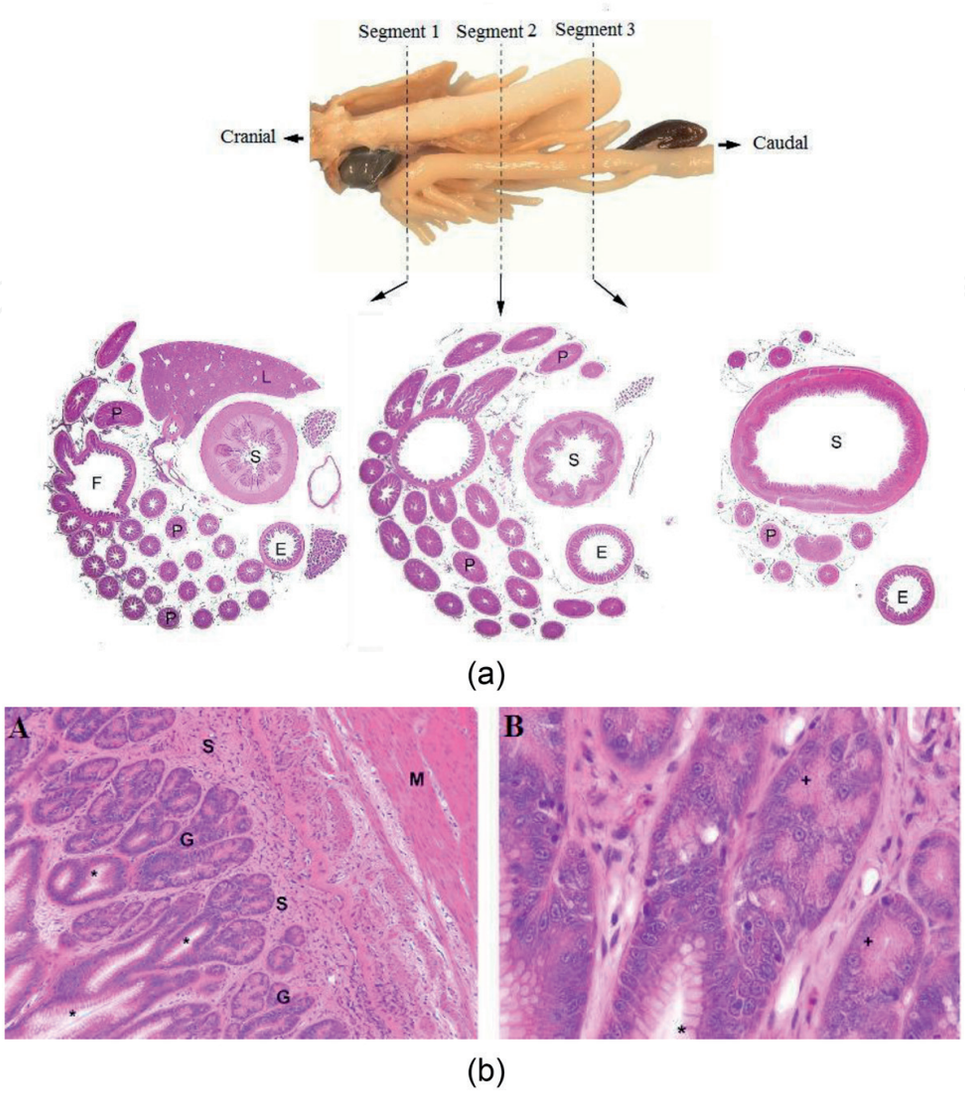

Figure 1. (a) On top: Different section-planes through the digestive tract of the rainbow trout. Below: Sections through segment 1, 2, and 3 consisting of Liver (L), stomach (S), foregut (F) with pyloric appendices (P), hind gut/rectum (E) (Source: [27]). (b) (A) rainbow trout stomach showing well-developed gastric glands (G), submucosa (S), and the tunica muscularis (M). (B) Higher magnification of branched tubular gastric glands. Asterisk (*) represents Shape and cellular morphology of mucous and (+) indicates pepsin and hydrochloric acid producing cell-type (oxyntopeptidic cells) (Source: [27]).

carnivores [39]. A reduction on feed intake and consequent decrease in weight gain is the first indicator that a given diet is exerting a negative effect.

There are several factors affecting the occurrence of SBMIE such as the inclusion levels, varieties, origins, and processing techniques of the different soybean products along with species variation and husbandry conditions (temperature, salinity, etc.). Nordrum et al. [40] who investigated the effect of salinity on the development of enteritis in salmonids. Urán [41] reported that with the increasing water temperature, the metabolic rate of Atlantic salmon increased which help to increase the severity of enteritis. Regarding the species variation effects on SBMIE, Nordrum et al. [40] also found that the effects of SBM on the intestinal morphology of rainbow trout were of less magnitude than for salmon. Similarly, Booman

et al. [42] reported that soybean meal induced enteritis in Atlantic salmon (*Salmo salar*) and Chinook salmon (*Oncorhynchus tshawytscha*) but not in pink salmon (*O. gorbuscha*).

To understand the mechanism of intestinal inflammation or enteritis, it is important to also understand the elements involved at the cellular level. Enterocytes are cuboidal shaped epithelial cells that are distinct on their apical surface than on their basal surface. The apical surface faces the intestinal lumen and shows the characteristic folding of intestinal cells, called microvilli. The basal side is connected to vasculature where absorbed nutrients are released. Nutrients can undergo transcellular transport on the apical and basolateral membranes of the cell; this can happen by diffusion or by active transport through transmembrane transporters like the glucose/Na+ cotransport system, or other amino acid transporters like glutamine transporter, and also through pumps and channels. Paracellular transport occurs in between epithelial cells, and only small molecules and ions, solutes, and fluids can reach the blood this way. Only small nutrients can diffuse this way, as, in healthy conditions, enterocytes are held together through important tight junctions that keep the intestinal integrity [31, 32]. Intercellular exchange between enterocytes is also possible though gap junctions and desmosomes. **Figure 2** showing the healthy and abnormal enterocyte condition in fish.

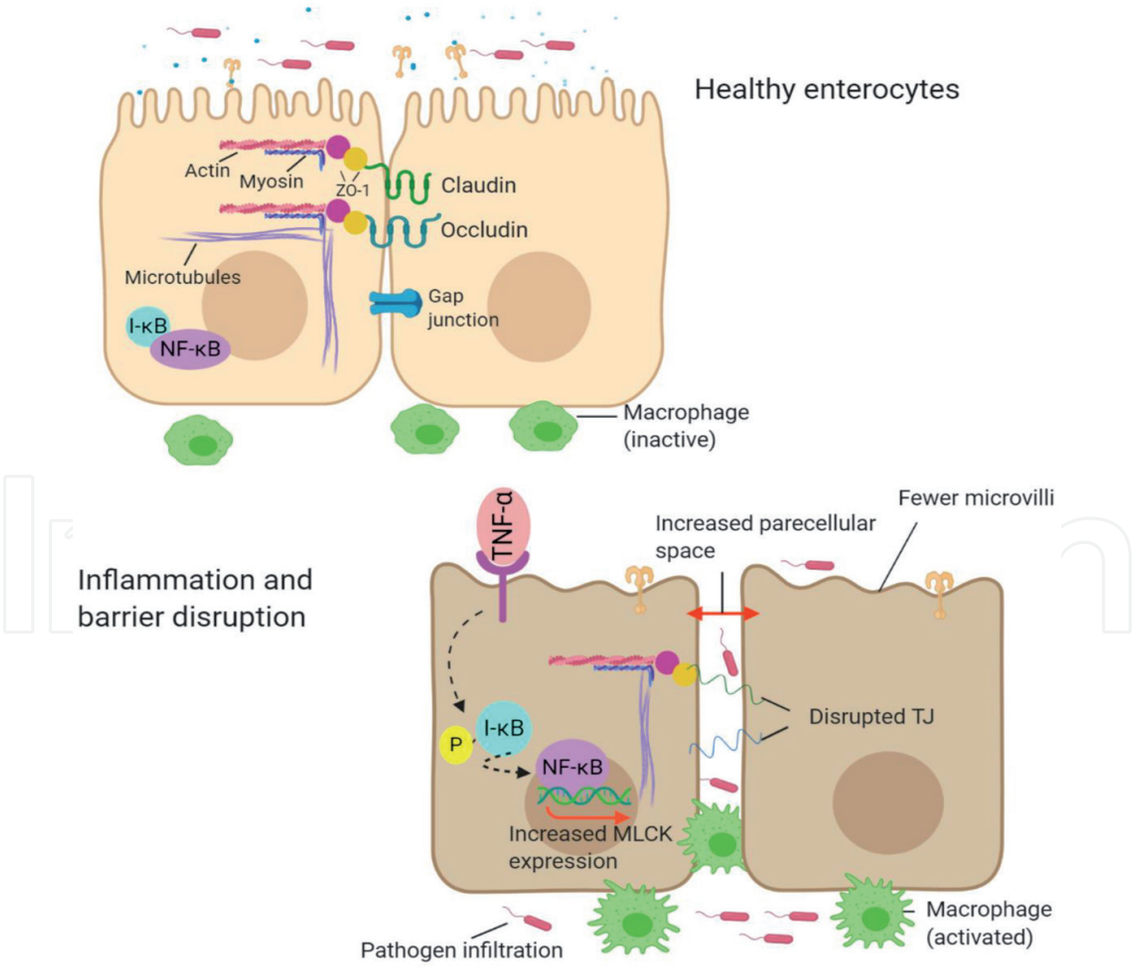

Figure 2. Figure showing the healthy and abnormal enterocyte condition in fish.

Some tight junction proteins that have been studied in fish with regards to SBMIE include transmembrane proteins like occludins and claudins and intracellular components like zonula occludens-1 (ZO-1) [26, 28, 31, 32]. Another intracellular component that interacts with the tight junction complex is myosin light chain kinase (MLCK), involved in cytoskeletal contraction, smooth muscle contraction, and, therefore, tight junction regulation and paracellular permeability [26, 43, 44].

3. Morphophysiological effects at fish gut of soy protein inclusion in aquafeed

Although soy protein has widely been used in aquafeed as a cheap alternative protein source for FM; however, the presence of some ANFs in SBM restricts its level of inclusion in aquafeed. High inclusion of soybean ingredients causes several negative effects on palatability and intestinal

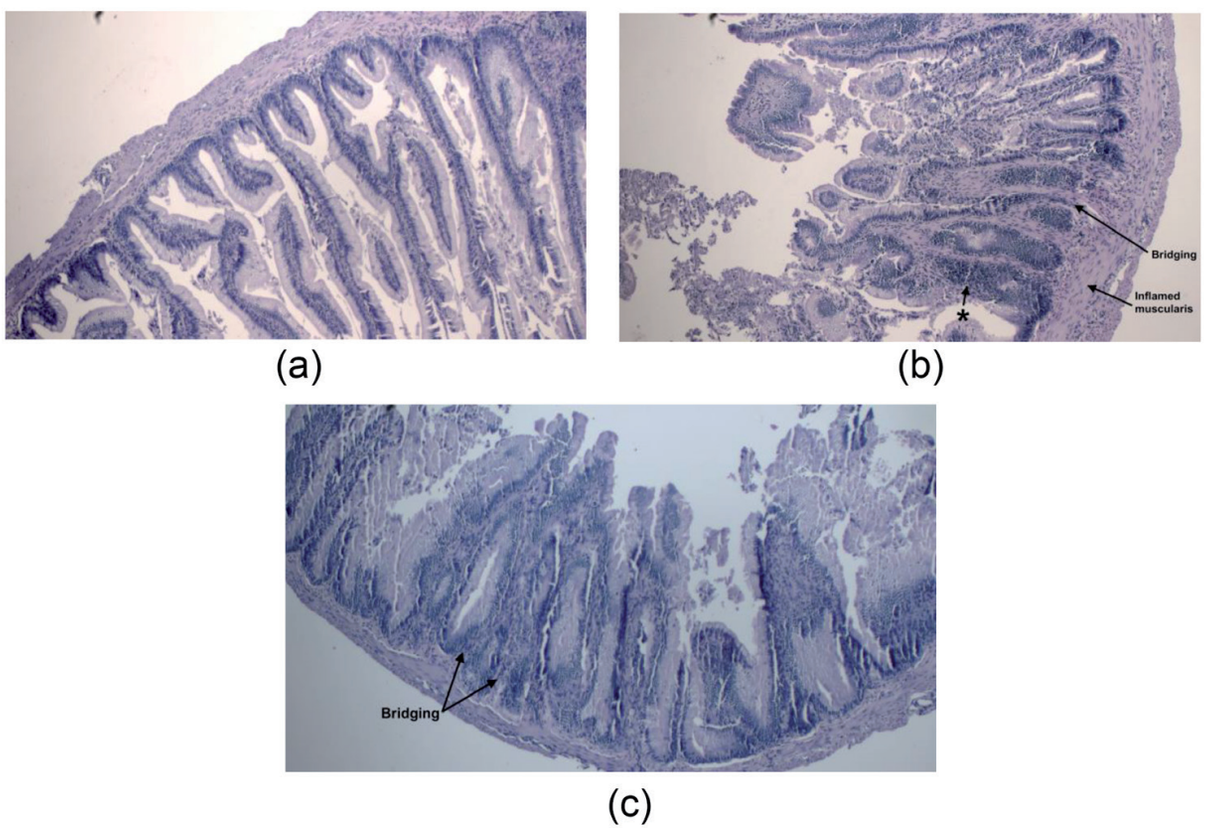

Figure 3. (a) Intestinal histology (posterior, intestine, 20 X) of rainbow trout (*O. mykiss*) fed FM-based control diet showing normal condition of intestine (Kumar et al., unpublished data). (b) Intestinal histology (posterior, intestine, 20 X) of rainbow trout (*O. mykiss*) fed low SBM (10.3%)-based diet showing inflamed muscularis, leukocyte infiltration of the lamina propria leading to swelling and mucosal fold fusion (bridging). Increased prevalence of globlet cells possibly to secrete more mucous to protect the epithelium. Asterisk denotes inflammation (Kumar et al., unpublished data). (c) Intestinal histology (posterior, intestine, 20 X) of rainbow trout (*O. mykiss*) fed high SBM (20.7%) based diet showing villi and lamina propria highly inflamed (leading to much wider mucosal folds), muscularis inflamed, villi shortened, disorganization of epithelium, reduction in supranuclear absorptive vacuoles, mucosal fold fusion (bridging) and some structural disintegration. By far, this treatment led to the most changes (Kumar et al., unpublished data).

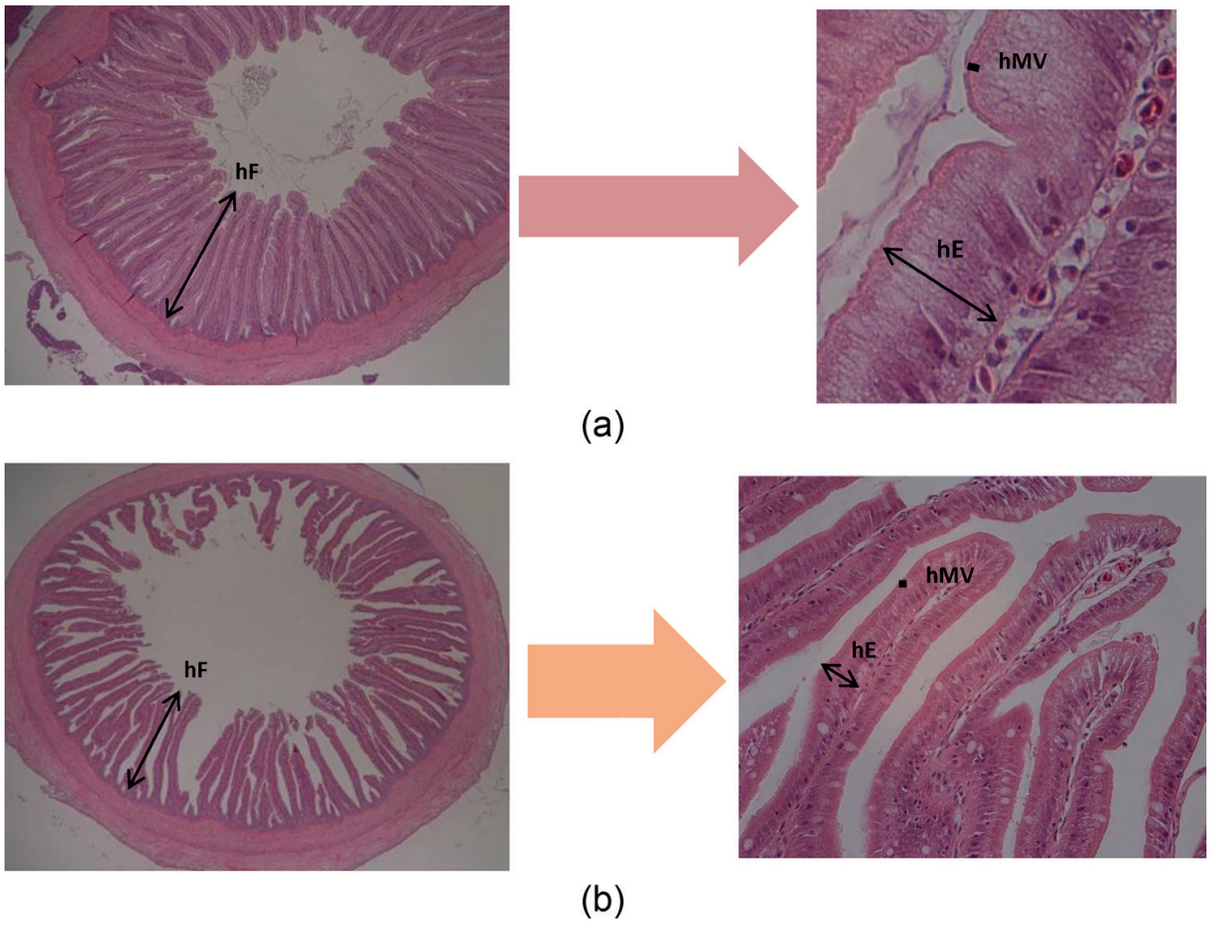

Figure 4. (a-1) Cross-section of anterior intestine (X 40, HE) of Amberjack (*Seriola dumerili*) fed FM-based control diet showing increased fold height (hF). (a-2) Cross-section of anterior intestine (X 400, HE) of Amberjack (*Seriola dumerili*) fed FM-based control diet showing increased enterocyte height (hE) and microvillus height (hMV). (b-1) Cross-section of anterior intestine (X 40, HE) of Amberjack (*Seriola dumerili*) fed 50% FM replaced with SBM-based diet showing reduced fold height (hF). (b-2) Cross-section of anterior intestine (X 400, HE) of Amberjack (*Seriola dumerili*) fed 50% FM replaced with SBM-based diet. Showing reduced enterocyte height (hE) and microvillus height (hMV).

morphology of fish. From previous researches, it is well documented that high inclusion of soybean meal (>40%) causes several intestinal morphological changes such as the reduction in mucosal folding, reduced fold height, enterocyte height, microvillus height, loss of mucosal integrity, abnormal vacuolization, and inflammatory cell infiltration (**Figures 3a–c** and **4a–1, a–2, b–1, b–2**) in aquatic animals [5–8, 36, 45–49]. The degree of morphological changes in the intestine depends on the inclusion level of SBM which is also correlated with the cultured fish species. Reduced fold height, enterocyte height, and microvillus height reduced the area of nutrient absorption in the intestine which finally affects the fish performances.

Feed nutrients must be digested for their utilization, and pancreatic digestive enzymes have essential roles for the digestion; trypsin and chymotrypsin are the main pancreatic proteases, lipase is the major pancreatic lipolytic enzyme, and amylase is known as the major pancreatic digestive enzyme for carbohydrates (Murashita et al. [50]). Inclusion of SBM that also affects the digestive enzyme secretion of different fish species is well documented. Murashita et al. [50] reported that red sea bream fed SBM showed lower content and activity of four pancreatic

digestive enzymes compared to fish fed FM. Also, lower gene expression levels of the digestive enzymes in the hepatopancreas were observed in the SBM fed red sea bream compared with the FM fed fish, which is in line with the report in yellowtail; orally administrated FM increased the trypsin and lipase gene expressions in the pyloric caeca, but not in fish administrated SBM [51]. Perera and Yúfera [52] reported that early SBM feeding of *S. aurata* larvae significantly affects the activity of most pancreatic enzymes in a time-of-exposure dependent form. More than 10 days of SBM feeding (i.e., beyond 14 dph) delayed the normal development of *S. aurata* larvae digestive capacities as the activities of all trypsin, chymotrypsin, and amylase were significantly reduced. This is opposed to the typical response of juvenile and adult fish to SBM. Protease inhibitors present in SBM can partially abrogate the activity of trypsin and chymotrypsin in the proximal intestine [53], and juveniles of *S. aurata* [54] and other fish such as Atlantic salmon [55, 56] exhibit a rapid compensatory increase in activities of these enzymes. However, SBM-induced increase in trypsin activity in juvenile fish is more marked in the distal intestine and has been attributed to a reduced ability to reabsorb the pancreatic enzymes [11] and the upregulation of trypsin-like activity by immune cells [56]. Therefore, other plausible explanation for our observations is that these intestinal processes are not fully functional in early larvae. Conversely, lipase activity was relatively insensitive to SBM in *S. aurata* larvae, as reported before in postsmolt Atlantic salmon [55]. The observed decrease in pancreatic proteases may be responsible for less lipase inactivation in the digestive tract explaining the stable lipase activity.

4. Soy protein inclusion impacts on gut-associated immunity

The gastrointestinal tract carries many functions in teleost; among them, defense is possibly one of the most important functions. Gut acts as a physical barrier to pathogen entry that also contains a gut-associated lymphoid tissue (GALT). Teleost gut-associated lymphoid tissue (GALT) consists of leucocyte populations located both intraepithelially and in the lamina propria with no structural organization. The gut microbes play a critical role in the development and maturation of GALT, which in turn mediate a variety of host immune functions [49]. Recent work on the structure of GALT and other intestinal cell populations, the absorption of macromolecules from the intestinal lumen, and the production of specific mucosal antibodies strongly suggests, however, that the gut of these lower vertebrates is immunocompetent. Fish intestine, especially the posterior segment, is immunologically active and armored with various immune cell types, including B cells, macrophages, granulocytes, and T cells [57, 58]. Studies on the gut-associated immunity are important for the aquaculture industry for several reasons. First, the gut is one of the main portals of entry of pathogens. Second, farmed fish are generally fed commercial pellets, which give farmers the ability to manipulate fish health by incorporating drugs, vaccines, and different feed ingredients or additives into the feed. Third, the gut immune system of teleost's allows microbial colonization by symbionts, and this microbial community can be regarded as a mechanism to modulate fish pathogens [59]. Many studies attempt to reveal the effects of inclusion of plant origin ingredients and different feed additives on gut-associated immunity in fish. In this current chapter, an attempt has made to discuss the effects of soy protein ingredients inclusion in aquafeed on gut-associated immunity of various fish species.

Research conducted on the modulation of gut immune response due to the inclusion of soy products in aquafeed predominantly concentrate on innate immune parameters. Several molecules involved in innate immunity are found in the intestine of different fish species, such as lysozymes in Asian sea bass (*Lates calcarifer*) [60] and Atlantic salmon (*Salmo salar* L.) [61]; complement components in grass carp (*Ctenopharyngodon idella*) [62], rainbow trout (*Oncorhynchus mykiss*) [63, 64], and Asian sea bass [65]; cytokines in Atlantic cod (*Gadus morhua*) [66] and rainbow trout [67]; lectins in several species (reviewed in [68]); or antimicrobial peptides (AMPs) in rainbow trout [69] and grouper (*Epinephelus coioides*) [70].

The immune status of a fish's intestinal mucosa is closely associated with inflammation, which is mediated by cytokines. Cytokines, such as IL-1, IL-10, and IL-16 have a fundamental role in the regulation of inflammatory responses in fish throughout the infection process [31, 32]. Many studies have shown that IL-1 and IL-16 are increased in inflammatory bowel disease and there was a positive association between disease activity [71, 72]. While the deprivation of IL-10 evokes the development of inflammatory bowel disease, the decrease of IL-10 can aggravates local inflammation [73, 74]. Wang et al. [75] reported the increased expression levels of IL-1 and IL-16 mRNA with the SBM level in the diet, whereas the IL-10 mRNA expression level decreased with the SBM level in the diet of orange-spotted grouper (*Epinephelus coioides*). At the same time, infiltrate leucocytes were observed in the intestinal epithelium in grouper fed diets contained SBM. Furthermore, the degree of intestine inflammation was positively correlated with IL-1 and IL-16 mRNA expression levels but negatively correlated with expression of IL-10 mRNA. Their results suggest that SBM can cause intestinal inflammation by increasing pro-inflammatory cytokine levels and decreasing anti-inflammatory cytokine levels.

Krogdahl et al. [35] examined the effect of solvent and alcohol extracted SBM in the diets of Atlantic salmon, and their results indicated that fish fed solvent-extracted SBM showed higher mortality rate when challenged by *A. salmonicida*. In addition, fish fed alcohol-extracted SBM revealed increased levels of both lysozyme and IgM in the mid and distal intestinal mucosa. In Atlantic salmon, Lilleeng et al. [76] showed significantly downregulated TGF-β gene expression after on day feeding of extracted SBM (460 g kg^{-1}), whereas reduced expression of interferon-inducible lysosomal thiol reductase (GILT) was observed followed by 3 days feeding. The authors assumed that the downregulation of TGF-β and GILT might be due to the failure to maintain mucosal integrity in the distal intestine. Sahlmann et al. [77] investigated transcriptomic profiling in Atlantic salmon feeding of SBM (200 g kg^{-1}) containing diet for 1 week. On days 3 and 5, a prominent change in gene expression patterns was observed. Immune-related genes were upregulated during the first 5 days: GTPase IMAP family members; NF-kB-related genes; and regulators of T-cell and B-cell function. These immune genes expression profiles suggest that intestinal inflammation is induced within a week upon administration of an SBM-containing diet, which may in turn negatively influence the growth performance of salmonids.

Bruce et al. [29] evaluated processed soybean meal ingredients (defatted soybean meal, bioprocessed soybean meal [BSBM], and commercial soy protein concentrate [CSPC]) inclusion in the diets of rainbow trout on intestinal immunity. They reported no significant differences in intestinal immunoglobulin concentrations ($p = 0.41$) or gut leukocyte phagocytosis at day 15 samplings ($p = 0.41$). Intestinal lysozyme activity showed some modulation throughout

the feeding trial period, with the BSBM diet producing higher levels in the long-term sample (60 day). A previous study on Atlantic salmon (*Salmo salar*) showed increased lysozyme activity in the intestinal mucosa due to the dietary inclusion of soybean molasses, indicating a potential inflammatory response and was potential activation of leukocytes [35]. Kim and Austin [78] also found high lysozyme activity in rainbow trout intestinal mucus samples after the administration of probiotics compounds which may be closely related to bioprocessed plant-based ingredients. Therefore, increased lysozyme levels may also be indicative of intestinal innate immunity and gut health enhancements.

The mucosal immune system in fish includes certain immunocompetent cells and factors in the intestinal mucous membrane. Of these factors, the interleukins (ILs), interferon regulatory factors (IRFs), and tumor necrosis factors (TNFs) are the main immune-relevant factors linked to inflammation in the distal intestine in fish [79]. Recently Miao et al. [80] reported the substitution effects of dietary SBM on the mucosal immune system in northern snakehead through measuring the gene expression of certain inflammatory cytokines (IL-1β, IL-8, IL-10, and IL-17F) in the distal intestine. After 63-day feeding, trial results indicated that dietary soybean meal affected the gene expression of certain factors. The up-regulated relative expression of IL-1β in the fish fed diet group containing 75% defatted fishmeal replacement with SBM was consistent with the observations in Atlantic salmon [77, 81]. However, the level of IL-1β observed in the same diet group was only 1.6-fold higher than that in FM-based control diet, while that observed in Atlantic salmon was 20-fold higher [79]. The effect of dietary soybean meal on the expression of IL-1β reflects the fish species and stages due to the different tolerance capability for soybean meal [79].

5. Effects of soy protein inclusion on gut microbiota

Healthy gut microbiota is essential to promote host health and well-being. Before the 1970s, there were some controversies regarding the existence and role of an indigenous microbiota in fish. However, it is now well established that fish and other aquatic animals have a microbiota in the GI tract (for review, see; [21, 23, 82–92]). The intestinal microbiota of fish, as is the case of mammals, is classified as autochthonous (indigenous) or allochthonous bacteria [90, 93]. The autochthonous bacteria are those able to colonize the host's gut epithelial surface or are associated with the microvilli, while the allochthonous bacteria are incidental visitors in the GI tract and are expelled after some time without colonizing [90, 93]. Several factors affect the gut microbiota in fish including host factors, environmental factors, microbial factors, etc. However, until recently, among different influencing factors affecting the fish microbiota, water and diet (environmental factors) have been studied extensively [49]. In this section, we address the effect of dietary soybean products on intestinal bacterial community of finfish and crustaceans (**Table 1**).

5.1. In salmonids

Research conducted until recently on SBM inclusion effects on gut microbiota of fish indicated that SBM modulated the intestinal microbiota toward developing an undesirable microbial community that can induce mucosal inflammation [110, 111]. Heikkinen et al. [94] reported that rainbow trout fish fed FM- and SBM-based diets for 4 weeks showed decreased number

Species/initial weight	Soy protein type and feeding duration	Effects on gut microbiota	References
Rainbow trout			
21.1 ± 1.4 g	SBM (450 g/kg) for 8 weeks	↓ culturable bacteria, *Lactobacillus* spp., *Sphingomonas* spp. ↑ *Bacillus* spp., *Chryseomonas* spp.	Heikkinen et al. [94]
~40 g	SBM (450 g/kg) for 16 weeks	→ total culturable aerobic levels, *Micrococcus* spp. ↓ *Aeromonas* spp., *Vibrio* spp. ↑ *Actinomycetales*, *Psychrobacter* spp., *Saccharomyces* spp.	Merrifield et al. [95]
1.56 ± 0.9 kg	SBM (300 g/kg) for 8 weeks	↑ no. of clones identified as *Carnobacterium maltaromaticum* ↓ no. of different sequences in library	Mansfield et al. [96]
~510 g	SBM (300 g/kg) for 8 weeks	↑ *Firmicutes*: *Proteobacteria* ratio DGGE analysis revealed low similarity indices between SBM- fed fish and the control	Desai et al. [97]
17.21 ± 0.51 g	defatted soybean meal (SBM), bioprocessed soybean meal (BSBM) and commercial soy protein concentrate (CSPC) replaced approximately 73% menhaden fishmeal and fed for 60 days	The incorporation of processed soy-based proteins alters the microbial community composition within the distal intestine. Species diversity based on abundance and evenness were lowest in the SBM group, and were significantly less than the BSBM-L ($p = 0.003$) and CSPC ($p = 0.003$) treatments	[29]
Atlantic salmon			
172 g	SBM (250 g/kg) for 3 weeks	↑ autochthonous bacteria in MI and DI, allochthonous in DI → no. of genera and strains ↑ *Brevibacterium*, *Enterococcus*, yeast ↓ *Marinilactobiacillus psychrotolerans*, *C. maltaromaticum*	Bakke-McKellep et al. [98]
242 ± 8 g	SBM (436 g/kg) for 4 weeks	→ viable counts ↓ *Carnobacterium* spp., *Bacillus* spp.	Ringø et al. [99]
1204 ± 34 g	SPC (50 g/kg)	↑ bacterial diversity, *Escherichia coli*, a Pseudomonadales	Green et al. [100]
144.5 ± 2.3 g	SBM (378 g/kg) for 35 days	→ total and viable counts ↑ *Aeromonas* Via, *Sporosarcina equimarina*	Navarrete et al. [101]
305 ± 69 g	SPC (200 g/kg) for 12 weeks	→ total autochthonous bacteria (proximal Aintestine; PI), total allochthonous bacteria, allochthonous community composition and total autochthonous bacteria (DI) ↑ autochthonous *Enterobacteriaceae*, Bacilli-like, *Lactobacillaceae*, *Streptococcaceae* in PI ↓ autochthonous *Vibrionaceae* in PI ↑ autochthonous Bacilli- like, *Streptococcaceae* in DI	Hartviksen et al. [102]
~133 g	SBM (200 g/kg) for 80 days	↓ the diversity indices in DI, *Weissella confusa* in the DI, proportion of Photobacterium in MI ↑ relative abundance of *Firmicutes* compared with the FM group, abundance of *Lactococcus lactis* subsp. Lactis in MI → Photobacterium in DI	Reveco et al. [103]

Species/initial weight	Soy protein type and feeding duration	Effects on gut microbiota	References
	SBM (246 g/kg) for 84 days	↑ allochthonous bacterial level in FG, HG → allochthonous bacterial level in HC ↓ autochthonous bacterial level in FG, HG and HC	Refstie et al. [47]
	SBM (246 g/kg) for 84 days	Modulated gut microbiota. ↑ *Chryseobacterium* and *Psychrobacter*	Ringø et al., [22]
~534 g	BPSBM (214 g/kg) for 84 days	→ population levels of adherent and allochthonous bacteria in FG, HG and HC Modulated gut microbiota. ↑ *Psychrobacter*	Ringø et al. [22]a
~24 g	SBM (313 g/kg) for 9 weeks	↑ species richness, Shannon- Weaver index	Dimitroglou et al. [104]
Ctenopharyngodon idella (weight not given)	SBM (13 g/kg) for 8 weeks	↑ *Pseudomonas putida* Aeromonas sp. DH69 *Actinobacterium bacilli* bacterium	Huang [105]
Carassius auratus ♀ × *Cyprinus carpio* ♂ (24.7 g ± 0.4 g)	SBM (300 g/kg) for 8 weeks	→ total culturable aerobic and anaerobic bacteria, presumptive *E. coli*, *Aeromonas*, Bifidobacterium, *Clostridium perfringens*	Cai et al. [106]
Carassius auratus (15 g)	SBM (355 g/kg) for 8 weeks	→ on gut microbiota determined by DGGE	Raggi and Gatlin III [107]
Three cyprinid species	SBM (40 g/kg) for 8 weeks	Modulation of the allochthonous gut microbiota. ↓ Proteobacterium clone (EF707282.1), *Cetobacterium somerae* (AB353124), *Bacillus subtilis*, *Anoxybacillus flavithermus*	Li et al. [108]
Oreochromis niloticus ♀ × *Oreochromis aureus* ♂ (~2 g)	SBM for 8 weeks	↑ *Plesiomonas* sp. BTOK4 *Aeromonas aquarium*	Zhang et al. [109]
Northern snakehead	FM replaced with graded level of SBM and fed for 63 days	At the phylum level, ↓ *Firmicutes* abundance was the lowest in the diet group having 75% defatted fishmeal replacement with SBM, ↑In contrast with *Proteobacteria*, *Bacteroidetes* and *Planctomycetes* ↓At the genus level, significantly lower abundance of Lactococcus, *Geobacillus*, *Pseudomonas*, *Streptococcus*, *Bacillus* and *Acinetobacter* in diet group (75% defatted fishmeal replacement with SBM) ↑ but higher abundance of *Cetobacterium*, *Planctomyces*, *Shewanella*, *Thermomonas*, *Rubrivivax* and *Carnobacterium* was observed in fish fed the same diet group (75% defatted fishmeal replacement with SBM)	Miao et al. [80]

Table 1. Effects of soy protein inclusion on gut microbiota of fish.

of cultivable intestinal bacteria (aerobic and anerobic). Afterward, by the 8 weeks of feeding trial, the bacterial numbers increased in the FM group, but not in the SBM group. Length heterogeneity analysis of PCR amplified 16S rDNA (LH-PCR) data also suggested a diet-related qualitative change in the intestinal microbiota of fish. The dominant identified genera were among aerobic species *Aeromonas*, *Sphingomonas*, and *Chryseomonas* and among the lactic acid bacteria, the genera *Lactococcus* and *Lactobacillus*. Rainbow trout fed SBM (450 g/kg) for 16 weeks showed decrease in total culturable species of *Aeromonas* spp., *Vibrio* spp., but the species *Actinomycetales*, *Psychrobacter* spp., *Saccharomyces* spp. were found as increased number. Total culturable aerobic levels, *Micrococcus* spp., were found unchanged in numbers. Mansfield et al. [96] evaluated the effect of FM and SBM (300 g/kg) on the allochthonous distal intestinal microbiota of triploid female rainbow trout by three cpn60 universal clone libraries, resulting in 1000 and 1181 sequences from FM and SBM, respectively. There were total 32 different sequences were noticed. The most frequently observed sequences were identical to *Carnobacterium (piscicola) maltaromaticum* and accounted for 55 and 97.2% of the clones from the FM and SBM group, respectively. Overall, fish fed FM showed highest diversity (14 different sequences) and only four different sequences observed in the SBM library. In another study, Desai et al. [97] observed that 30% SBM inclusion in rainbow trout diets led to a reduction in *Proteobacteria* and increase in *Firmicutes*. Recently, Bruce et al. [29] evaluated different processed soybean products as a replacement of fishmeal on gut microiota of rainbow trout and observed that the incorporation of processed soy-based proteins alters the microbial community composition within the distal intestine. Species diversity based on abundance and evenness were lowest in the defatted soybean meal group and were significantly less than the bioprocessed soybean meal in low concentration ($p = 0.003$) and commercial soy protein concentrate ($p = 0.003$) treatments.

In Atlantic salmon, fish fed the SBM (250 g/kg) diet had higher total number as well as a more diverse population composition of adherent bacteria in the distal intestine observed by Bakke-McKellep et al. [98]. Green et al. [100] investigated the influence of FM and soybean protein concentrate (SPC; 50 g/kg) on intestinal microbiota of Atlantic salmon. Terminal restriction fragment length polymorphism (T-RFLP) and 16S rRNA clone library analysis revealed that the SPC diet modulated the intestinal microbiome by increasing the bacterial diversity, and a *Pseudomonadales* was more frequently revealed species. In addition, increased *Escherichia coli* also observed in SPC-based diet, but it was absent in FM-based diet. In another study, Navarrete et al. [101] reported SBM supplementation (378 g/kg) effects on distal intestine microbial community of Atlantic salmon. Principal component analysis (PCA) revealed correlations that fish fed SBM diet was correlated with *Aeromonas* VIb and *Sporosarcina aquimarina*, while *Microbacterium*, *Pseudomonas*, *Lactococcus lactis sp*. cremoris, and *Aeromonas* VIa were correlated with the FM-based diet. Reveco et al. [103] investigated the microbiota in the mid and distal intestine of Atlantic salmon fed FM and solvent extracted SBM (200 g/kg) by DGGE analysis. Results showed increased *Lactococcus lactis* subsp. lactis in the mid-intestine, while a reduction in *Weissella confusa* in the distal intestine of Atlantic salmon fed 20% solvent extracted SBM-contained diet. Hartviksen et al. [102] revealed no dietary effect of soy protein concentrate (SPC) on total autochthonous bacteria isolated from PI and total allochthonous and total autochthonous bacteria isolated from DI of Atlantic salmon by qPCR analysis. However, significant ($p = 0.05$) effect was observed regarding community composition. An increase was noticed in autochthonous *Enterobacteriaceae*, Bacilli-like, *Lactobacillaceae*, and

Streptococcaceae in PI, and Bacilli-like and *Streptococcaceae* in DI by SPC feeding. In contrast, a significant ($p = 0.05$) decrease was revealed in *Vibrionaceae* in PI.

5.2. In cyprinid fish

Most of the literature available on the effects of different soy products on the gut microbiota are on salmonid fish, and less is known for other species. The possible reasons behind this might be due to the less susceptibility of non-salmonid fish to SBMIE and histological damage [39]. In cyprinid fish, like in grass carp, the effects of dietary SBM inclusion (1.3% by dry weight) were compared with the inclusion of casein meal (CM; 1.0% by dry weight) on the autochthonous gut microbiota [105]. After 8 weeks of feeding, 16S rRNA PCR-DGGE analysis revealed a clear difference between the microbiota of the SBM group and the CM group with similarity between the groups of only 26% ($p < 0.05$). Unique bacteria isolated from the CM group were identified as follows: uncultured *Lachnospiraceae bacterium*, uncultured *Lactobacillus*, uncultured *Clostridium* spp., and uncultured *Proteobacterium*, while bacteria isolated from the SBM group were identified as *Pseudomonas* sp., *Aeromonas* sp., uncultured bacteria, uncultured *Actinobacterium*, and uncultured *Bacillus* spp.

Raggi and Gatlin [107] evaluated four probiotics diets based on FM and SBM on gut microbiota of goldfish (*Carassius auratus*). After 8 weeks of feeding, denaturing gradient gel electrophoresis (DGGE) analysis results revealed no difference in gut microbiota. The probable reason explained for this observation is due to the incorporation of dietary chromic oxide (10 g kg^{-1}) which may have reduced the quantity and complexity of the bacterial community as reported by Ringø [112] for Arctic charr (*Salvelinus alpinus* L.). Cai et al. [106] also reported no significant effects of fishmeal replacement by SBM (30%) on the levels of total aerobic bacteria, total anaerobic bacteria, presumptive *E. coli*, *Aeromonas*, *Bifidobacterium*, or *Clostridium* in the intestine of silver crucian carp (*Carassius auratus gibelio* × *Cyprinus carpio*). Recently, the effect of partial replacement of SBM (4%) by intestinal casing meal (ICM), prepared from the wastewater of enteric coating and heparin processing, was used to evaluate the effect on the allochthonous gut microbiota of three cage-cultured cyprinid species [108]. Results indicated that the allochthonous bacterial diversity was altered by ICM substitution; however, by feeding ICM, some bacterial species were significantly stimulated, *E. coli*, and *Exiguobacterium* in black carp (*Mylopharyngodon piceus*) and species belonging to *Firmicutes*, Fusobacteria, and *Proteobacteria* in gibel carp (*Carassius gebelio*).

5.3. Cichlids and others

The effects of replacing dietary SBM or cottonseed meal (CSM) by completely hydrolyzed feather meal (CHFM) on the composition of gut microbiota was investigated by Zhang et al. [109] for hybrid tilapia. After 8 weeks of feeding, 16S rRNA PCR-DGGE analysis results revealed that CHFM induced modulation of the whole intestinal microbiota in hybrid tilapia and prevented colonization of potentially harmful species in the intestinal tract. *Plesiomonas* sp. BTOK4 and *Aeromonas aquarium* were found in decreased level in diet group where 120 g kg^{-1} CSM was replaced with CHFM. Miao et al. [80] reported the substitution effects of dietary SBM on the intestinal microbial community of northern snakehead. After 63-day feeding, trial results indicated that dietary soybean meal substitutions significantly affected

the intestinal microbiota composition of fish. At the phylum level, *Firmicutes* abundance was the lowest in the diet group having 75% defatted fishmeal replacement with SBM, in contrast with *Proteobacteria*, *Bacteroidetes*, and *Planctomycetes*. At the genus level, significantly lower abundance of *Lactococcus*, *Geobacillus*, *Pseudomonas*, *Streptococcus*, *Bacillus*, and *Acinetobacter*, but higher abundance of *Cetobacterium*, *Planctomyces*, *Shewanella*, *Thermomonas*, *Rubrivivax*, and *Carnobacterium* was observed in fish fed the same diet group (75% defatted fishmeal replacement with SBM).

From previous research, it is established that gut microbiota influences several physiological and immunological aspects of aquatic animals like development, digestion, nutrition, immunological functions, and disease resistance [113, 114]. The gut microbiota together with digestive enzymes, mucins, peristalsis, and epithelial barrier with tight junctions belongs to the so-called non-immune component of mucosal immunity [115]. Moreover, several previous research findings indicated that intestinal microbiota is required for full immune maturation [116, 117], inflammatory diseases [117, 118], and to increase the host's resistance toward pathogenic invasion and infection [119]. However, until recently, research relating on the effects of soy protein inclusion in fish feed and their interaction among gut microbiota and immune responses is scarce. So, further research on the interaction effect on gut microbiota and innate immune system due to soy protein utilization are required for further confirmation of the usability of SBM.

6. Conclusion

The highest maximum exploitation of marine resources used to produce FM has enforced fish nutritionist to use alternative protein sources as FM substitute in aquafeed. Worldwide availability and relatively cheaper price make SBM as one of the suitable alternative ingredients in aquafeed. However, high proportion of soy protein sources inclusion in aquafeed may impair fish immunity, maturation, and functionality of the intestinal mucosa, the first line of defense, and damage the gastrointestinal tract. However, using the appropriate proportions of alternative protein sources as well as SBM provides not only the option of limiting harm, but also there is also an interesting possibility to enhance GI immunity and disease resistance. From the available literature, it is showed that non-salmonids are less susceptible on the effects of SBM on the gut microbiota as well as the gut health than salmonids species. Until today, research on the effects of high soy protein inclusion in non-salmonid diets on gut health is little; so, more research warranted for non-salmonids fish. Future study is also needed on the use of different functional supplement in SBM-based diet to increase the efficiency of utilizing alternative protein (soy protein) through maintaining improved physiological and gut health condition. To date, most of the studies on SBM inclusion in aquafeed and its effects on fish intestinal microbiota were descriptive and only concerned the composition of the microbial community. Further works are warranted to investigate the functions of subpopulations in the microbiota and ultimately the functions to the species level due to alternative protein inclusion in aquafeed. In addition, the anaphylactic effects of SBM and the immune regulatory mechanisms involved merits further investigation.

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Conflict of interest

The authors declare no conflict of interest.

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