AIMS Animal Science, 1 (1): 65–148.
DOI: 10.3934/aas.2025006
Received: 12 April 2025
Revised: 17 July 2025
Accepted: 22 July 2025
Published: 15 October 2025

https://www.aimspress.com/journal/aas

Review

Responses of rainbow trout to total replacement of fishmeal by proteins from single cells and plants: A review

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Abstract: Globally, production and value of rainbow trout exceeds 1 million tonnes and $5 billion annually. Due to its value as a sport and food fish the species is cultivated on every continent, other than Antarctica, in all types of culture system (ponds, raceways, tanks, RAS, cages and net pens) and, as a founding species of contemporary aquaculture, aquarians of trout were some of the first to face many of the technical and sustainability challenges that still plague the industry today. One of the more pressing issues, especially for high trophic species, is their reliance on fishmeal (FM), derived from reduction or forage fisheries, as feed ingredients. Feed represents the largest production cost for trout producers and the most substantial contributor to nutrient discharges and environmental impact. Several methods, including ingredient substitutions, have been employed in efforts to reduce the overall ecological effects of trout feed while reducing dependence on the finite and unpredictable supplies of small pelagic species. Consumers too have become progressively more aware of the negative ecological and social impacts of forage fisheries which has encouraged industry and feed manufacturers to search for FM alternatives with the eventual goal of its complete removal from feeds. As we move towards an increasing variety of novel ingredients that aim to shake the dependence of trout cultivation upon its FM addiction, research will no doubt intensify. Studies undertaken to date have examined a broad variety of alternative proteins including those with single-cell organisms, terrestrial and aquatic plants, and a range of animals and their processing byproducts. However, the existing literature is widely dispersed and often difficult to access. The present text takes account of plant and single cell-derived proteins that have been used for the complete replacement of FM and reviews their effects on physiological control processes, while pointing to areas worthy of future study.

Keywords: alternative proteins; growth; quality; immunity; reproduction; gene expression; microbiome

List of abbreviations

3n: triploid; ANF: antinutritional factors; AP: animal protein; APD: apparent protein digestibility; BePC: bean protein concentrate; BM: barley meal; BOD: biological oxygen demand; BP/C: barley protein/concentrate; BSCP: bacterial single cell protein; CAP: Clostridium autoethanogenum protein; Cas-Gel: casein-gelatin; CG/M/F: corn gluten/meal/feed; COD: chemical oxygen demand; CPC: corn protein concentrate; CPI: canola protein isolate; CSC: cottonseed concentrate; CSM: cottonseed meal; CSPP: cottonseed protein powder; DDGP: distiller's dried grains; d/RPC/I: dephytinized/rapeseed protein concentrate/isolate; (d)/(e)/(f)/(se)SBM: defatted, enzyme-treated, fermented, or solvent extracted soybean meal; (E)AA: (essential) amino acid; ERE: energy retention efficiency; EPM: extruded pea meal; EWW: extruded whole wheat; FAA: free amino acids; FB(PC): faba bean (protein concentrate); FC: feed consumption; F(C)E: feed (conversion) efficiency; FCR: feed conversion ratio; FE: feed efficiency; FF: first feeding; FFS full-fat soybean; FI: feed intake; FM: fishmeal; FPC: fish protein concentrate; fSBM: fermented soybean meal; GDDY: grain distiller dried yeast; GIT: gastrointestinal tract; GM: guar meal; GSI: gonadosomatic index; HG: high glycoalkaloid; H/LDCP: high/low dicalcium phosphate; HSI: hepatosomatic index; IPFR: intraperitoneal fat ratio; K: condition factor; Leu: leucine; LG: low glycoalkaloid; LS: linseed; LSM: lupin seed meal; Lys: Lysine; Met: methionine; MGM: maize gluten meal; MHA: methionine hydroxy analogue; Mg: magnesium; Mn: manganese; MNM: mixed nut meal; MP: microplastic; MR: muscle ratio; N: nitrogen; NT: nucleotide; OMP: Oregon moist pellet; P: phosphorus; PCB: polychlorinated biphenyls; PeM: dehulled pea meal; PePC/I: pea protein concentrate/isolate; PE(R): protein efficiency (ratio); PI: protein intake; POP: persistent organic pollutants; PP: plant protein; PRE: protein retention efficiency; PS: polystyrene; PSP: pistachio shell powder; PSM: pea seed meal; PNM: peanut meal; PPC: potato protein concentrate; PPV: protein fixed/protein intake; PR: protein retention; PRO: Profine®; RC: rice protein concentrate; RDDG: rice derived distillers grain; RLM: red lentil meal; RS: rapeseed; RSM: rapeseed meal; SBM: soybean meal; SBME: soybean meal extract; SBTI: soybean trypsin inhibitor; SCP: single cell protein; SF: soy flour; SPI: soy protein isolate; SM/C: sunflower meal/concentrate; SGR: specific growth rate; SPC: soy protein concentrate; Suppl.: supplementation; TAN: total ammonia nitrogen; Tau: taurine; TGC: thermal growth coefficient; Thr: threonine; VSI: viscerosomatic index; WF: wheat flour; WG/M: wheat gluten/meal; WM: wheat middlings; WLM: white lupin seed meal; wt: weight; WW: whole wheat; Y/E: yeast/extract.

1. Introduction

There is an embarrassing wealth of information on the dietary replacement of fishmeal (FM) in aquafeeds [reviews: *e.g.,* 1–30]. They include studies that have replaced FM using single cell, vegetable and animal proteins of terrestrial and marine origin, both with and without nutritional additives, palatants, and other ingredients. Experiments have been undertaken using various species

of farmed fish to examine partial through total dietary FM substitution, with most reporting on the effect of such manipulations upon growth, feed conversion efficiencies (FCE) and changes to body composition. These investigations have also provided valuable information that enhances our understanding of how fish respond to dietary manipulations in terms of changes to their digestive physiology, health and immune function, gene expression, reproductive potential, and product quality. Studies with alternative proteins have also considered technological aspects of FM substitutions such as physical pellet quality, mechanical stability, durability, pellet disintegration, particle size distribution, and impacts on effluent water quality [*e.g.*, 31–42]. Several reviews have focused on salmonids and dietary FM replacement [*e.g.*, 43–51] with some concentrating on nutrient densities of alternative proteins and their limitations, their essential amino acid (EAA) profiles and effects on growth, the presence of antinutritional factors (ANFs), and animal health, especially associated with alterations in the gastrointestinal tract (GIT).

The use of alternative proteins such as fly larvae and plant byproducts, exemplified by shipstuff or wheat middlings, as ingredients for salmonid fry feeds has been practiced since at least the late 1800s [52–55], continued into the 1900s [56,57] and remain in use through to the present [58,59]. Like today, although not necessarily directly articulated, there was an aspiration to develop trout culture into a more sustainable venture as early as the 1910s. Concerns at the time included reducing water pollution caused by using raw meats (fish, horse, seal, sheep) and offal (liver, spleen, heart, lungs) in diets. Concurrently, culturists sought to confront problems related to storing fresh and frozen feedstuffs, while developing diets that were more easily disseminated [60–66]. Thirdly, and especially during war years, fish and meat were rationed and/or becoming more expensive [67–69]. Thus, cheaper plant-based feeds increased in appeal. Among other issues, there was a growing understanding of disease transmission from forage to cultured fish and the nutritional requirements of trout [70–72]. Early studies with diets that did not include either liver or kidney resulted in poorer growth and health issues which were assumed to occur because of vitamin deficiencies [73,74]. Preliminary studies with animal protein-free diets with various trout species also reported changes in physiology, inferior growth and FCEs, and increased mortalities [57,75]. These adverse reactions were generally attributed to plant-derived toxins and nutritional inadequacy and were so commonly described that some suggested use of plant meals, especially in fingerling feeds, was inadvisable [76].

It was not until the mid-1940s that test diets were developed that enabled determination of vitamin requirements of rainbow trout (the Wisconsin diet [77]) with their ultimate refinement allowing quantitative determination of EAAs, being resolved [78–80]. In the intervening years, various dietary formulations were evaluated. For example, pelleted feeds, such as the Oregon diet (OMP [see 81]), and dry preparations, began to be used experimentally in state salmonid hatcheries and at commercial farms in the 1950s [82–85]. At around the time of their introduction, however, epizootic levels of hepatoma were reported [86,87]. Although recognized as a pathology in rainbow trout as early as 1909 [88], and only sporadically noted thereafter [89,90], suspicions were directed towards feed components as the causative agent. The presence of adventitious toxins and ANFs, including carcinogens and mutagens derived from molds, thus became more methodologically investigated. For hepatomas this ultimately led to a specialized conference held in 1965 [91].

Throughout the 1950s various trials were undertaken using innovative ingredients. One of the first appraisals of yeast and penicillin mats – the dried ground mycelia of *Penicillium* spp. used for antibiotic production - in trout feeds was undertaken by [92], and [93] examined the utility of torula yeast as a vitamin source, while [94] provide an early suggestion for FM-free mash. Grassl [95],

compared the growth of rainbow trout fed either wet chopped meats or dry pelleted animal/vegetable feeds, supplemented with beef liver every 3 weeks as a vitamin source. He reported identical growth even when the pellet was fed at 50% the amount recommended for raw feeds and observed that trout fed diets containing brewer's yeast grew better than those fed the same diet but having torula yeast – perhaps an early indication of a probiotic effect for live yeast. Implementation of the pelleted feeds across all Michigan's state hatcheries resulted in 60% improved production and a 40% reduction in feed costs. Use of a vitamin mixture in the dry pellet, as recorded by [96], ended the need for chopped liver supplements and synchronously formed the basis for trout dry pellet formulations and development of mechanized feeders [97–99]. As pointed out by [79] in their comprehensive review of the history of feed progress, the future expansion of global aquaculture will depend on advances in alternatives for FM proteins. This is because the majority (62.3%) of traditional fisheries operate at or just below maximum sustainable yields, with 37.7% being over-fished [100]. These data do not consider illegal and unreported fishing, nor at-sea discards or inaccuracies in data collection such that the situation for world fisheries may be even more dire. In fact, the use of FM in salmonid diets has declined substantially since the mid-1990s [*e.g.*, 101] and this trend is set to continue. One of the main reasons for adjustments to dietary formulations has been the increasing costs of FM associated with the declining catches from industrial fisheries. The latter has accrued due to over-fishing, natural (*e.g.*, El Niño–Southern Oscillation, Madden-Julian Oscillation events) and climate-induced effects, and, among others, increasing human consumption of forage fish [102,103]. In addition, wealthier and better-informed consumers have started to become more aware of lifestyle diseases, the environmental impact of fisheries, different farming practices and animal welfare issues, each of which may be contemplated before making food purchase decisions [104,105].

A considerable number of studies have examined the replacement of FM with various alternative proteins in rainbow trout diets. Fewer have evaluated the effects of complete substitutions, and these are outspread in the literature. In this review an effort is made to connect these widely dispersed sources. Only studies where *complete* dietary removal of FM is reported are considered. This does not weaken the importance of the many findings that have used partial or even substantial FM substitutions but acts to consolidate the considerable library of information available. Many of the latter communications served to highlight significant issues that needed surmounting to achieve the goal of complete FM elimination from trout feeds. Herein we consider removal of FM by single celled (SCP) and plant (PP) proteins only. The impact of substituting FO by a wide variety of plant oils is considered elsewhere [*e.g.*, 106–110].

2. Search methods

The current review was constructed using various search engines, including Google and Semantic Scholar, Web of Science, Scopus and other electronic catalogs. The citation listings for each included paper, back issues of relevant journals and publisher's websites, key authors' web pages when available, proceedings volumes, meeting abstracts and reference to various existing bibliographies [111–116]. No language restrictions were used during any of the searches.

3. Candidate proteins

"The supply must be convenient and certain; the cost must be such as not to entail too great an expenditure for the value of the crop of fish; it should be a substance of easy and rapid preparation, and, above all, the chemical composition, or proportion of nitrogenous and nonnitrogenous constituents, should be in accordance with the requirements of the fishes to be fed."

[53]

Conventionally, the main PPs employed in aquafeeds have been derived from legumes, oilseeds and cereal grains, and thorough accounts of the variety evaluated are presented in [117]. Comparative compositional analyses and apparent digestibility coefficients (ADCs), of variously processed PPs for trout are contained in [118], the USDA [119] and [120] databases. Issues surrounding their availability, palatability, production, pricing, and ease of handling, shipping, storage and regulatory constraints are emphasized by Gatlin and colleagues [3,26]. As noted by Page in the quotation above, alternative ingredients must also be straightforward to use during feed production, and ideal candidates should express relatively high protein content, a favorable amino acid (AA) profile, high digestibility and good palatability, together with low quantities of fiber and starch.

Most plants have several biologically active ingredients that have apparently evolved to protect them from being consumed. However, these bioactives may be both beneficial (*e.g.*, antioxidants, immunostimulants, prebiotics) and detrimental when ingested as food. In context with the current review, it is the negative effects that are more germane, and these include the capacity of the various ANFs (Table 1) to reduce palatability, decrease feed efficiencies and create nutrient imbalances, impacting bioavailability and ultimately leading to poorer growth. ANFs have been proven to cause intestinal dysfunction, and to alter the gut's microbiome and are known to disrupt immune function, be engaged in goitrogenesis, trigger pancreatic hypertrophy and hypoglycemia and, among others, to invoke hepatic disorders [121]. The specific class(es) of ANF within legumes, oilseeds and cereals (Table 1), their potency, and diversity vary among varieties [3]. Mechanisms of actions of various ANFs and their effects on fish are considered in [121–124], and include protease inhibition, negative impacts on vitamin and mineral utilization, immunological and reproductive stress. For example, plant protease inhibitors bind to trypsin resulting in the enzyme's inactivation [125] and consequent reduced protein digestibility. Plant trypsin inhibitors, such as that from soybean, can also modify the form in which proteins are absorbed, increasing the uptake of intact proteins and polypeptides [126]. By binding to vitamins, saponins inhibit their absorption [127] and negatively impact protein digestion and intestinal patency [128,129]. Phytic acid reduces the availability of a number of ions and phytic acid phosphate has poor digestibility [130] resulting in the need to supplement feeds with Phosphorus (P). This leads to loss of P to the aquatic environment, leading to development of harmful algal blooms and decreased oxygen levels. Phytoestrogens have been isolated from over 300 species of plants [131] and may impede estrogen uptake and bind to receptors regulating reproduction. Phytoestrogens also affect feed intake and impact protein synthesis and increase protein degradation in trout [132]. For most ANFs, the severity of their effects appears to be dose, developmental stage and interaction-dependent.

The negative effects of many, but not all ANFs, can be reduced or eliminated using various methods of processing including milling, soaking, heat treatment, germination, fermentation, chemical and enzymatic transformations, solubilization, precipitation, γ irradiation, supercritical fluid and

pulsed electric field technologies, *inter alia* [130,133–138]. For example, lectins, which interfere with nutrient absorption and may cause hemorrhages, leading to reduced growth, are heat-labile and damaged by heat treatments. Oxalates, which hinder mineral uptake, can be countered through feed enrichments of calcium while saponins can be removed by soaking. Increased mineral and phosphate digestibility from phytic acid-containing plant products can be enhanced through treatment with phytases [130]. The aforementioned procedures reduce the presence or eliminate ANFs and have resulted in improved fish performance especially with plant concentrate- and isolate-based feed formulations (Table 2). Other approaches include marker-assisted plant breeding programs that help in developing varieties that express naturally low levels of antiproteases, glucosinolates, and others [139–141]. However, since many of the ANFs play critical roles in plant physiological control processes, for example, phytic acid is engaged in P storage and chelation of micronutrients for growth and development, this is not a trouble-free proposition. More recent have been trials with GMO and genome edited crops that try to modify specific genomic sequences to moderate the influences of antiproteases and other ANFs, including influencing the environmental quality of soluble discharges. For example, antiproteases and phytate in soybeans can increase P emission [121] and hence elevate the ecological footprint of discharges.

Table 1. Commonly employed alternative protein sources, their global production, major antinutritional factors (ANFs) and reported production impacts on rainbow trout [after 121,122].

Crop	Tonnage	Major ANFs	Main impacts
Barley	154,877,140	1-3, 7, 16, 23.	↓ growth
Corn/maize	1,163,497,300	1-2, 6-7, 23.	↓ growth
Canola/rape	87,221,224	1-2, 4-5, 7, 23.	↓ growth, digestibility
Cottonseed	41,617,340	2, 6-9, 19, 23.	↓ growth
Faba bean	4,839,721	1-3, 5, 7, 10, 23.	↓ growth, FCE
Lupin	1,644,691	1, 6, 11-12, 23.	↓ growth, digestibility, FI
Pea	14,166,030	1-3, 5, 13, 16-17, 19, 23.	↓ growth, digestibility
Peanut	50,714,000	1-3, 6, 11, 16, 23.	↓ growth
Potato	374,777,760	1-2, 6, 11-12, 14, 16-17, 20-21, 23.	↓ growth
Rice	776,461,440	1-3, 7, 16, 23	↓ growth
Soybean	349,856,420	1-4, 6-7, 11, 16, 19, 23	↓ growth, FCE
Sunflower	50,000,000	1, 7, 11, 15, 20, 22-23.	↓ growth
Wheat	808,441,600	1-3, 18, 20, 23.	↓ growth

Key: 1. Antiproteases, 2. Phytic acid, 3. Lectins, 4. Glucosinolates, 5. Tannins, 6. Phytoestrogens, 7. Aflatoxin potential, 8. Gossypol, 9. Cyclopropenoic acid, 10. Glycocides, 11. Saponins, 12. Alkaloids, 13. Cyanogens, 14. Oxalate, 15. Arginase inhibitor, 16. Phytohemagglutinin, 17. Cyanogen, 18. Flatulence factor, 19. Anti-vitamin E factor, 20. Amylase inhibitor, 21. Invertase inhibitor, 22. Arginase inhibitor, 23. non-starch polysaccharides. FI = feed intake.

SCP, which include bacteria, yeasts, fungi and algae, have been used as feed ingredients for various trout at least since the 1950s [92]. SCPs have garnered increased interest as aquafeed ingredients because they do not need extensive areas of land for production and are water-sparing compared to

traditional crops. In addition, some processes used for growing algae and bacteria use CO_2 and NH_4, respectively, and are thereby considered as positives for climate change. Moreover, unlike conventional crops, SCPs can be produced, independent of climate and seasonal constraints, year-round. In recent decades innovative technologies have greatly increased SCP production and down-stream processing capacity, and a bonus is that nutrient recovery and bioconversions of agricultural byproducts, restaurant, supermarket and institutional discards, coincidentally save on landfill [23]. Several reviews provide coverage of the production technologies employed to generate SCP biomass and the variety of SCPs employed in growth trials with various cultured species [*e.g.*, 7,8,23,142–144].

Table 2. Example reported effects of total FM replacement with plant proteins on the growth response of rainbow trout.

Start size, days, temp	Main proteins examined*	Response of fish relative to FM-based feeds	Ref.
19 g, 84-d, 15°C	SBM, SPC, CPC, WGM, WW, +/- 0-2% PSP	↑ wt gain and SGR ($P \leq 0.02$)	[306]
35 g, 56 d, 15°C	PNM, CGM, CF, CGF	↓ wt gain, FI and PER ↑ FCR ($P < 0.05$)	[307]
	SBM, CGM, CGF, CF	↓ wt gain and PER ↑ FCR, PER ($P < 0.05$)	
	SPCs, CF, CGF	↓ wt gain, FI and PER ↑ FCR, PER ($P < 0.05$)	
	SF, CGM, CF, CGF	↓ wt gain and PER ↑ FCR, PER ($P < 0.05$)	
19 g, 84 d, 17°C	CGM, WGM, EPM, RSM	↓ wt gain, FE, SGR, and PER ($P < 0.05$) ↑ FI ($P < 0.05$)	[308]
33.6 g, 25 d, 16.5°C	LSM, CGM, SBM, WG, WW, PeM	↑ growth, FI and SGR ($P < 0.05$)	[309]
104 mg, 240 d, 17°C	RSM, PSM, CG, LSM	↓ wt gain, FE and FI ($P < 0.05$)	[310]
6.1 g, 70 d, 11°C	FSP	↓ wt gain ($P < 0.05$; 176% v 336% gain) ↑ FCR ($P < 0.05$)	[295]
33.6 g, 36 d, 11°C	FSP	↓ wt gain ($P < 0.05$; 69% v. 83% gain) ↑ FCR, APD ($P < 0.05$)	[299]
23.4 g, 205 d, 11°C	FSP	↓ wt gain ($P < 0.05$) ↑ FCR ($P < 0.05$)	[269]
15.9 g, 94 d, 11°C	FSP, CG	↓ wt gain ($P < 0.05$) ↑ FCR ($P < 0.05$)	[272]
38 g, 86 d, 14.5°C	RC, SPC, BPC WGM, CGM, SBM	↑ FCR	[164]
4.8 g, 105 d, 14.5°C	SPC, CGM, WG, SBM	↑ survival but ↓ growth in PP diets ($P < 0.05$) ↑ FCR and feed intake in PP diets ($P < 0.01$)	[294]
4.8 g, 105 d, 14.5°C	SPM, SBM, CGM, WG,	↓wt gain and % protein retention ($p< 0.05$) ↑ FCR, feed intake ($P < 0.05$)	[305]
104 mg 238 d, 17°C	WW, CG, WG, PeM, LSM, RSM	↓ wt gain, FE and FI ($P < 0.05$)	[312]
37 g, 84 d, 15°C	SBM, SPC, CPC	Flax/corn oil = FO for wt gain, FI and FCR DHA-Gold @ 90 mg g^{-1} ↑ wt gain, FI and FCR v. FO ($P < 0.04$)	[313]
125 g, 112 d, 15°C	SBM, CGM, SI, WW	↓FCR ($P < 0.03$)	[314]
125 g, 112 d, 15°C	SI, CGM, SBM, WW	No differences in wt gain, FI or FCR	[315]
10.6 g, 105 d, 13°C	SPC, SBM, CGM, WGM	↑ growth with PP diets ($P < 0.001$) Compared two strains	[316]
26 g, 168 d, 17°C	WG, CG, SBM, SPCWLM, RSM, PeM	No differences in growth parameters measured	[317]
12 g, 84 d,	SPI, WF, CG,	↑ FI, FCR ($P < 0.001$), ↓ growth, PRE ($P < 0.001$)	[247]

14.5°C			
140 mg, 164 d, 11.4°C	WW, CGM, SBM, WLM, PeM	↓ growth and FE ($P < 0.01$),	[318]
140 mg, 318 d, 17°C	CGM, WG, SBM, SPC, WLM, PeM, WW	↓ growth	[319]
84 g, 53 d, 11°C	SBM, WGM, CGM + Met/Lys	Fish fed PP diet were leaner ($P < 0.05$) Depression in growth and FCR	[286]
0.4 g, 197 d, 11.4°C	CG, WG, SBM, WLM + RS, LS, Palm oils	↓ growth ($P < 0.001$) ↓ FI and FE ($P < 0.01$)	[320]
<100 mg, 35 d, 11.4°C	WW, SBM, WLM, WG, CGM, FB	↓ growth ($P < 0.01$)	[321]
0.5 g, 189 d, 11.4°C	WW, FB, CG,WG, SBM,WLM, PeM	↓ growth ($P < 0.05$)	[322]
82-92 g, 84 d, 17±1°C	FBPC, PPC, WG, CG, SBM, GM	Compared different parental nutrition (FM v. PP) Offspring from high carb, low protein diet had higher feeding rate ($P < 0.05$) and whole-body lipid content ($P < 0.006$)	[323]
Fry, 32 d, 16°C	CG, SBM, PePC, SPC, BePC	↑ growth for progeny from PP-fed broodstock	[324]
9.2 g, 56 d, 14.5 °C	SBM, WG, CG, WW +/- MHA	↓ growth ($P < 0.001$), ↑ FCR ($P < 0.001$)	[325]
4 g, 50 d, 14°C	SBM	↓ growth, FI and SGR ($P < 0.05$)	[326]
1 g, 177 d, ambient ~14°C	SBME	Growth inhibition and mortality ↓ absorption of Leu, Met and Thr	[327]
246 g, 224 d, 11-16°C	CSM	↓ growth ($P < 0.05$)	[328]
214 g, 84 d, 13.1°C	SPC, CPC, SBM	No differences in wt gain, TGC or *K*.	[31]
0.14 g, 210 d, 7°C	MGM, WGM, WLM, SBM, PM, PeM, RSM	↓ weight and length gain ($P < 0.01$) but *K* similar, HSI ($P < 0.001$), VSI ($P < 0.05$).	[302]
162 g, 3n, 157 d, 17.1°C	CGM, WG, EPM, RSM	wt gain, SGR, PER and FE inferior in PP fed fish ($P < 0.05$)	[267]
1.2 g, 63 d, 10°C	WLM, CGM, WG, WW, PeM, SBM	↓ growth, FI, FE ($P < 0.001$) ↑ mortality ($P < 0.001$)	[229]
106 g, 90 d, 18°C	SPC	↓ wt gain ($P < 0.006$) No diff. in *K*	[285]
39.5 g, 180 d, 18°C	SPC, WG, SBM, CG, WM	No diff. in wt gain, FI and *K*	[329]
0.1 g, 100 d, 17°C	SBM, PePC, CG, WW	↓ wt gain, SGR ($P < 0.05$) ↑ FCR	[330]
42 g, 84 d, 17.5°C	WG, CGM, SPC, SBM, WLM, RSM, PeM, WW	No differences in any measured growth parameter with increasing dietary levels of Se	[331]
4.7 g, 70 d, 15°C	FAA -based diets	↓ growth, SGR ($P < 0.05$) for all diets	[332]
20 g, 84 d, 15°C	CGM, WGM, SBM, SPC +/- Met/Lys/Gly/Thr	Wt gain highest in feeds with Met/Lys/Thr[a] = Met/Lys/Gly[a] = Gly/Lys/Thr/Met[a,b], no suppl.[b,c] Highest FCR in fish with no suppl. ($P < 0.01$)	[244]
26.8 g, 63 d, 15°C	PRO, CGM, WGM, SBM, + Tau	↓ wt gain ($P < 0.05$) in all PP-based feeds Tau suppl. ↑wt gain, PRE and ERE v PP-diet without Tau ($P < 0.05$) ↓ FI lower in 0.5-1.0% Tau levels v. PP-diet without Tau ($P < 0.05$)	[255]
18.4 g, 84 d, 15°C	CGM, WGM, SBM, SPC +/- Tau/Met	Met suppl. ↓ growth ($P < 0.01$) Tau suppl. @ 5/10 g kg^{-1} ↑ growth ($P < 0.005$) No effect of Tau/Met on FCR but FC was reduced	[257]

		(P < 0.055)	
72 g, 84 d, 15ºC	SBM, CPC, SPC, +/- Lys, Thr	↓ wt gain (P < 0.05) in all PP-based feeds Lys/ Thr suppl. ↑ wt gain v. non-suppl. PP diets (P < 0.05) and for first 2 formulations, growth equivalence with FM and AP diets.	[333]
	CPC, SPC, DDGP, +/- Lys, Thr		
	CPC, *Spirulina*, BPC, +/- Lys, Thr		
33.5 g, 16.5ºC	WLM, CGM, SBM, WW, PeM	↓ growth (P < 0.05)	[334]
29 g, 147 d, 15 ºC	LSM, CG, SBM	↓ growth, SGR, FI (P < 0.05)	[335]
16.2 g, 70 d, 14-16 ºC	FSB, SBM, CGM	↓ growth, PER (P < 0.05)	[336]
54.5 g, 56 d, ~15ºC	LSM, FB, PSM, CG, SBM	↓ growth, SGR, FI (P < 0.05)	[337]
19.2 g, 77 d, 16-18ºC	CGM, WG, RSM, EWW, EPM	↓ growth and FI, FE, SGR, PER, N retention (P < 0.05)	[338]
1.17 g, 60 d, 14.5ºC	Enzyme-treated SBM	↓ growth, SGR, FI (P < 0.05) ↑ FCR (P < 0.05)	[339]
130 mg, 240 d, 15°C	WM, CGM, WGM, PeM, PM, RSM, WLM	Monosex RBT; ↓ growth, SGR, FI (P < 0.05)	[340]
~18.9 g, 84 d, 15ºC	CPC, SBM, SPC, WGM,	↓ growth, SGR, FI (P < 0.05)	[341]
35 g, 56 d, 12-14ºC	CAP, CPC, SPC, WG, CGM, MM	↓ growth and SGR (P < 0.05) ↑ FCR (P < 0.05)	[279]
46 g, 150 d, 14 and 18 ºC	SPC, SBM, WLM, WG, Y, S*pirulina* + camelia oil	No effect of temp on growth but lack of FM increased FI and FE ($P \leq 0.03$)	[342]
39.5 g, 150 d, 14-20ºC	SPC, SBM, CG, WF, RS, LSM, Y	14 ºC ↓ growth, SGR, FI (P < 0.001) 18 ºC ↓ growth, SGR, FI (P < 0.001) 20 ºC ↓ growth, SGR, FI (P < 0.001)	[343]
15 g, 60 d, 14-15ºC	WG, CGM, SBM	↓ growth, SGR and *K* (P < 0.05) ↑ FCR (P < 0.05)	[235]
16.5 g, 112 d, 15±1°C	SCP, SBM, WW	↓ growth, FI, PER and PPV (P < 0.05) ↑ FCR (P < 0.05)	[344]
83 g, 84 d, 15±1°C	SPC	No differences in wt gain, FE or PER	[345]
37.5 g, 60 d, 13ºC	PeP, SBM, WM	↓ growth, PER, SGR, and FCR (P < 0.001)	[346]
24/156 g, 28 d, 17ºC	SPC	↓ growth, PER, FI (P < 0.05) in 24 g fish ↓ growth an FI (P < 0.05) but no difference in PER in larger trout	[347]
121 g, 84 d, 18ºC	MGM, SBM, WG, RS, LS, Palm oils	↓ growth FE, SGR and FI (P < 0.05)	[348]
~0.5 g, 1-2 y, ambient	CGM, SBM, WG, WLM, + RS, LS, Palm oils	↑ wt (P < 0.05) of 2 yr olds, maybe due to restricted rationing of FM diet Wt identical in 3 yr olds.	[234]
23.7 g, 343 d, ambient	SBM, WG, MGM, WLM	↓ growth	[349]
31.5 g, 84 d, 14.5ºC	CG, SPC, WGM, LDCP CG, SPC, WGM, HDCP	↑ FCR, PER NPR, P retention (PR) ↓growth, SGR (P < 0.05) ↑ PR ↓ NPR, FI (P < 0.05)	[350]
25.3 g, 70 d, 15ºC	RPC, PSM, WG, CGM + NT	↓ wt gain (P < 0.05) ↑FCR (P < 0.05)	[351]
39 g, 56 d, 15ºC	CSM, SBM, WG	↓ wt gain, FCE (P < 0.05)	[352]
14.7 g, 90 d, 14ºC	CGM	↓ wt gain, SGR (P < 0.05)	[353]

106 g, 90 d, 18°C	SPC + Met	↓ wt gain ($P < 0.001$) Addt. 2.2 or 4.2 mg kg^{-1} Met ↑wt gain vs SPC with no suppl. Met ($P < 0.05$)	[354]
~0.1 g, 30 d, 17°C	SBM, PePC, CG, WW	↓ wt gain, FI ($P < 0.01$) FE, survival	[355] [356]
11.7 g, 84 d, 16°C	fSBM, SBM, CGM	↓ wt gain ($P < 0.003$) ↓ FE and FI ($P < 0.005$)	[291]
~975 g, 33 d, 16°C	SPC + Met 1.26-2.23 g/16g N	wt gain lower for all SPC-based feeds ($P < 0.05$) wt gain was poorest in lowest Met suppl. level ($P < 0.05$) SGR, FI, digestible energy/protein retention lower ($P < 0.05$)	[357]
~10 mg dry wt, 11.6°C, 75 dph	PePC, RPC, SPC, WG, CGM	↓ wt gain ($P < 0.05$)	[358]
250 mg, 93 d, 11.6°C	PePC, RPC, SPC, WG, CGM	↓ wt gain ($P < 0.05$)	[359]
40 g, 45 d, 15°C	Cas	Weight and SGR equivalent, ↓ FI, ↑FCR ($P < 0.05$)	[360]
2.4 g, 42 d, 15°C	SBM	↓ wt gain, PER, FCR ($P < 0.05$)	[361, 362]
32 g, 56 d, 15°C	SBM, LSM, CGM, PPC	wt gain, SGR, FI no difference ($P > 0.05$)	[229]
18.4 g, 70 d, 16°C	e/fSBM, FSP, CGM	↓ wt gain, SGR, FER ($P < 0.05$) ↑FI ($P < 0.05$)	[293]
19.8 g, 90 d, 15°C	SBM, SI, +/- tau	↓ wt gain, SGR, FER ($P < 0.01$)	[363]
4.3 g, 167 d, 15°C	BM, WG, CG, SBM	↓ wt gain for first 76 d ($P < 0.05$), but equivalent thereafter	[268]
33 g, 84 d, 15°C	SPC, CPC, WG, SBM	↑ wt gain and SGR for selected strains	[364]
5 g, 180 d, 15°C	WGM, CPC, SBM, SPC, BM		[365]
10 g, 60 d, 12.3°C	CGM, RLM, WG	↓ wt gain, SGR and K ($P < 0.05$) FCR equivalence	[366]
5 g, 252 d, 15°C	SBM, SPC, CPC, WF, WGM	↓ wt gain ($P < 0.05$)	[367]
121 g, 66 d, 17°C	CGM, SBM, WG. RS, LS, Palm oils	↓ wt gain and FE with PP ($P < 0.00005$) ↓ PER ($P < 0.007$), DGI ($P < 0.002$) and FI ($P < 0.003$)	[368]
FF, 364 d, 17°C	WLM, CGM, WG, EWW, PeM	↓ wt gain, PER and FE (≤ 0.01)	[369]
162.5 g, 160 d, 17°C	Unidentified PP	↑ *K* ($P < 0.05$); ↓ growth, ($P < 0.05$)	[370]
19 g, 157 d, 17°C	PP-based	↓ growth ($P < 0.05$)	[371]
622 g, 365 d, 8-10.7 °C	SBM, seSBM	↓ FCR, no difference in wt	[233]
130 mg, 90 d, 10 °C	CG, FB, SBM, PP, WG, GM, SPC, RSM	↑ FI, ↓ wt gain, ↑ FCR ($P<0.05$)	[372]
78.1 g, 56 d, 17.5°C	CGM, WG, SBM, SPC, WLM, PeM, RSM, WW	↑ FI, FCR ($P < 0.05$) ↓ growth, PER ($P < 0.05$)	[373]
36.5 g, 84 d, 17°C	CGM, WG, SBM, SPC, WLM, PeM, WW	↑ ADC for P, Mg and Cu ($P < 0.05$) ↓ ADC for K ($P < 0.01$) ↑ FI, ↓ growth	[374]
19.8 g, 84 d, 17°C	CGM, WG, SBM, SPC, WLM, PeM, RSM, WW	↑ FCR ($P < 0.001$) ↓ FI, growth, FE ($P < 0.001$)	[375]

19.8 g, 84 d, 17°C	CGM, WG, SBM, SPC, WLM, PeM, RSM, WW	↓ growth, FI, FE ($P < 0.05$) Apparent availabilities of Mg, K, Fe, Cu, Mn, Zn lower	[376]
914 g	Commercial PP feed	↓ FI, growth, FE ($P < 0.02$)	[377]
19.5 g, 98 d, 15°C	SPC, CPC, SBM + Lys/Met/Tau/ Thr/Cu + $CuSO_4$/$ZnSO_4$	↑ wt gain ($P < 0.05$) ↓FCR ($P = 0.001$) Addt. of Zn ↑ wt gain ($P < 0.008$)	[300]
28.6 g, 126 d, 15°C		Addt. Of $CuSO_4$ was without effect No impact of Cu/Zn on FCR	
49.1 g, 84 d, 17°C	WG, PeP, FBPC, SBM, RSM, YE	↓ FI and SGR with ↑ dietary YE ($P < 0.05$)	[276]
223 g, ~112 d, 9.6-21.6°C	CSM	↓ growth ($P < 0.05$)	[378]
49.1 g, 84 d, 17°C	SPC, RSM, FB, WG, PePC	No differences in wt gain or SGR FI and FCR ↑ ($P < 0.05$)	[379]
2.6 g, 182 d, 9.2°C	SPC SBM	↓ wt gain ($P < 0.05$) ↓ wt gain ($P < 0.05$)	[380]
19.2 g, 84 d, ~17°C	CGM, WG, PeM, RSM, WW	↓ wt gain ($P < 0.05$)	[125]
71 g, 100 d, 11.5°C	SBM, WG, CG, PePC	No differences in wt gain, FCR, SGR or TGC. Fecal stability was lower ($P < 0.05$) in the PP-based diet.	[155]
1.75 g, 60 d, 16°C	SPC, RC, CPC	No differences in growth, FI, SGR or survival	[381]
79.1 g, 84 d, 15°C	SPI, +/- Met/Gly Thr/Lys/Car	SPI-based diets resulted in ↑ FCR, ↓ growth ($P < 0.05$) SPI + AA and CSN equal to FM-based feeds SPI and SPI-AA feeds ↓ MR and ↑ IPFR ($P < 0.05$)	[248]
7 g, 150 d, 13.3°C	Cas-Gel	Addition of phytic acid and Ca-Mg mix resulted in ↓ wt gain ($P < 0.05$) v OMP	[382]
117 ±1.6 g, 49 d, 14°C	PePC, SPC, WG	↓ wt gain and SGR following restricted feeding ($P < 0.05$). No difference after satiation feeding.	[383]
12 g, 70 d, 15.5°C	SPC, WW	↑ FCR ($P < 0.05$) ↓ wt gain ($P < 0.05$)	[384]
4.3 g, 84 d, 10.2°C	dRPC, WM	↑ FI ($P < 0.05$) ↓ PER, FE, SGR ($P < 0.05$)	[385]
23.23 g, 63 d, 14.5°C	SPC, WG, CG, SBM, RSM, WM	No differences in growth, SGR or FCR	[281]
52.1 g, 84 d, 13.4°C	LG-PPC, HG-PPC	↓ growth, SGR, PER, PPV ($P < 0.05$) ↑ FCR ($P < 0.05$)	[277]
13.4 g, 70 d, 15.5°C	WM, WG, SBM	↓ growth, SGR, PER, ($P < 0.05$) ↑ FCR, FI ($P < 0.05$)	[278]
5 g, 60 d, 15°C	BM, WG, CGM, SBM + Lys/Tau	SGR lower ($P < 0.015$) in fish not selected for PP diets FCR poorer ($P < 0.024$) in fish selected for PP diets when fed FM-based feed	[280]
41 dph, 355 d, 13.9°C	MG, SBM, WG, WW, WLM, PeM	↓ growth, FI, FE ($P < 0.01$)	[386]
19.2 g, 84 d, 17°C	RSM, EPM, WGM, MGM	↓ growth, SGR ($P < 0.05$)	[243]
12 g, 84 d, 14.5°C	SPC, WF, CG	↓ growth, FCR, PRE, SGR ($P < 0.0015$)	[387]
23 g, 84 d, 14.5°C	SPC, SBM, CPC, WF	↓ growth, SGR ($P < 0.05$) ↑ FCR	[388]

20 g, 84 d, 14.5°C	MNM, SPC, WGM + Mn	↑ wt gain in PP diets suppl. with 2-8 mg kg^{-1} Mn v FM ($P < 0.05$) FI and PER identical Survival equal except for 32 mg kg^{-1} Mn which was lower ($P < 0.05$)	[389]
125 g, 84 d, 14°C	SPC, SBM, fSPC, CPC, BMA, PM	↓ growth and SGR for fish fed high SBM-based diet ($P < 0.05$) ↑ FCR for fish fed high SBM-based diet ($P < 0.05$)	[42]
5 g, 300 d, 12.5°C	CGM, SPC, WGM + Lys/Met	↓ wt gain ($P < 0.05$) FCR lower ($P < 0.05$) No impact of differing densities on performance	[284]
4 g, 42 d, 14°C	PPC, WF	↓wt gain, SGR, PER, FE, PER, PPV, mortality ($P < 0.05$)	[236]
10.4 g, 70 d, 16°C	FSP, CGM, SBM + EAA	↓wt gain, SGR, FE, PR ($P < 0.05$)	[256]
14.6 g, 70 d, 16°C	SBM, CGM, WF fSBM, CGM, WF	↓wt gain, SGR, ($P < 0.05$) ↓wt gain, SGR, FER ($P < 0.05$)	[304]
15.7 g, 70 d, 16.4°C	SBM, CGM, WF fSBM, CGM, WF +Met and Lys/+ EAA	↓wt gain, SGR, FER, PR ($P < 0.05$) ↓wt gain, SGR, FER, PR ($P < 0.05$) with ↑FI ($P < 0.05$) v. FM ↑ wt gain, SGR, FER, PR v. base PP diets alone but not v. FM Survival unaffected across all groups	[249]
208 g, 23 d, 10°C, 3n	SBM, SPC, CGM, WG CSPP, WM	No effect on wt gain	[390]
1 g, 189 d, 17°C	CG, WG, SBM, SPC, WLM, PeM, WW	↓wt gain, SGR ($P < 0.001$)	[296]
FF, 168 d, 11°C	CG, WG, SBM, SPC, WLM, PeM, WW	↓wt gain ($p < 0.05$)	[391]

3.1. Technical features of contender proteins

Irrespective of the production system used, water quality and its maintenance are critical for trout aquaculture. Poor operational water quality can affect fish physiological control processes and welfare which can lead to reduced growth and feed efficiency (FE), and elevated susceptibility to disease and, during severe challenges, mortality. The importance of controlling water quality has become especially relevant with water reuse or recirculating aquaculture systems (RAS). Alternatives to FM must not only achieve nutritional balance, but they must also express high digestibility and palatability while possessing low levels of ANFs, starch and fiber. ANFs not only impact nutrient digestibility but cause diarrhea that decreases fecal stability [39] and raises water column particulates that may result in gill damage and upregulation of IFN-γ genes in lamellae [145–147]. From a technical perspective, FM alternatives should also convey certain physical characteristics. For example, PP extrudates should demonstrate a certain level of porosity that allows assimilation of oil, while also being stable under storage and during pneumatic feeding [148]. Soybean meal (SBM), perhaps the most widely used alternative protein, satisfies a number, but not all, of the technical characteristics needed. Other than its high protein content and well-balanced AA profile, SBM-derived proteins and peptides, depending on their processing, have been shown to possess antioxidant, antimicrobial, and other beneficial properties, such as their gelling and emulsibility, and oil-holding capacity [149–151]. Nonetheless, previous studies with rainbow trout have demonstrated that a consequence of replacing FM with SBM and other PPs is increased, less stable fecal waste of smaller particulate size associated

with elevated total ammonia, biological oxygen demand (BOD) and water discoloration [42,152–155]. Because smaller-sized particles have a larger surface area-to-volume ratio they are more prone to leaching and thereby increase the load of dissolved organic matter into the water column. Moreover, smaller particulates may act to increase bacterial burdens by providing a substrate for heterotrophic growth [146,156]. As a rule of thumb, the smaller the size of particle, the more difficult and expensive it is to remove in terms of energy, instrumentation and water cleansing systems required.

Particulate removal systems may suffer from reduced efficiencies with increased particulate burdens and, in flow-through systems, such as ponds and raceways, smaller fecal particles also disperse over further distances and thus increase the area over which solid waste impacts the environment. Stocking density too may affect particulate sizing because of increased water turbulence due to swimming/fin movements and aggression, which underlines the necessity for speedy removal of all fecal waste. The design of in-tank fecal collection, concentrating, treatment, and removal components thus also becomes an important engineering consideration, especially for RAS [157–160], as does the production of diets that yield intact and settleable fecal solids that can be efficiently removed from the culture environment. Intriguingly, irrespective of system, most particulates express ellipsoidal, rather than rounded or elongated structures, differing only in ferret diameter [161]. These flake-like constructs are believed to be regulated by feed composition rather than holding system which may be relevant to system design, but comparisons between PP, SCP and FM-based diets apparently remain unstudied.

Speed of removal of fecal particles is of the essence since they express thixotropy – becoming less viscous and thinning over time due to shear forces from water flow. Curiously, medium sized trout (~150 g) void mechanically more stable feces than smaller (~40 g) and larger (~650 g) individuals [162]. Addition of guar gum to diets improved the overall mechanical stability to a greater extent in medium and large animals with a corresponding reduction in post-filtration effluent loading. Welker *et al.* [39] observed that addition of guar gum to a trout PP diet significantly reduced the production of fines while increasing production of larger fecal particles ($P < 0.05$) findings confirmed by [155]. In another study, Welker *et al.* [40] examined the effects of different alternative proteins, with and without guar gum as a potential stabilizing agent, on fecal particle size. They found that there was a significant effect of protein source on particle size, with larger sizes being prominent in those diets containing sardine and menhaden meals, and fines being prevalent in diets containing soy protein concentrate (SPC) and SBM. Interestingly, the diet containing corn protein concentrate (CPC) yielded similar particle size ranges as the FM-based feeds. Addition of guar to the SPC feeds increased the percentage of large-sized particles but decreased fines relative to SPC without guar ($P < 0.05$), with the former being like that produced by FM-based diets and the latter being significantly less than observed with FM feeds. Corresponding to the decrease in fines was a heightened level of mid-sized particles. The authors concluded that the addition of guar gum at 0.3% could negate the effects of soy-based diets on fecal particle makeup by improving the elastic modulus and viscosity of the feces, thereby enhancing fecal stability and thixotropic durability. Levels of dietary guar gum > 0.3% are not recommended, however, since this may interfere with nutrient digestion and absorption [163].

Zettl *et al*. [38] examined the effect of different mixes of PP ingredients upon the mechanical properties of pellets and determined that soy protein isolate (SPI) returned the most promising results when compared to camelina and rapeseed meal (RSM). Pellet durability was influenced by the binding properties of the individual proteins, their moisture content, compaction pressure and

temperature employed during pelleting with the latter acting to breakdown and, during cooling, reconfigure protein bonds. Martin *et al*. [37] compared a FM-based pellet with that of a rape seed press cake (RPC) feed and how the physical characteristics of each was influenced by extruder barrel temperatures and screw speeds. RPC did not affect extrusion system parameters but did, at temperatures evaluated (90, 100, 110 ºC), impact expansion indices (decreased versus FM, $P < 0.05$) which, in turn, influenced bulk density (decreased) and pellet hardness. The expansion index and bulk density also decreased with increasing screw speed (200–400 rpm). Barrows *et al*. [164] examined different extrusion barrel temperatures and pressures and the effect of pre-cooking on a FM/SBM (~1:2) feed. They found that pre-cooking reduced trypsin inhibitor activity, protein dispersibility and nitrogen solubility indices and improved ADCs for organic matter, carbohydrate and energy ($P < 0.02$). The authors recommended a barrel temperature of 127 ºC and residency of 18 s for highest weight gains and lowest FCRs. Further advantages of extrusion technologies have been notable reductions in trout farm environmental loadings in terms of suspended solids, BOD, COD, TAN, nitrites and nitrates and P outputs [165].

3.2. Safety issues

It is the potential negative impacts of FM contaminants that partly fuel the opinions of proponents for aquafeed ingredient substitutions. Nonetheless, alternative terrestrial plant replacements also come with some risks associated with chemical contaminants [26]. For example, legumes and cereals can become contaminated because of natural phenomena exemplified by mycotoxinogenic fungi and heavy metals, or due to anthropogenic activities (*e.g.*, application of herbicides and pesticides; *via* soil, aerial and water pollutants). The accumulation of various elements, such as As, Cd, Cr VI, Hg, Pb and Sn by plants may be contingent on the type and species of metal, their soil concentrations, and duration and frequency of exposure. Biomagnification of toxicants in plants can also be influenced by climate, soil type, and agricultural practices as well as the plant species. Thus, any discussion on the use of alternative proteins in aquafeeds, irrespective of their origin or type, must consider the risks that they may present relative to that of FM. It is well established that fish can bioaccumulate various chemicals ingested with food or *via* diffusion across the gills and skin from the surrounding environment. The speed of accumulation varies with contaminant concentration and form and may be further influenced by a variety of biotic and abiotic factors such as fish size, age, health, nutritional status, and prevailing temperature. The potential for biomagnification of noxious chemicals from feed both in nature and on farms has been demonstrated with numerous species, as has the contamination of aquafeeds by various chemicals. Feeds can be tainted with persistent organic pollutants (POPs) and heavy metals, natural toxins, and others. For example, Berntssen *et al.* [166] detected dioxins and dioxin-like polychlorinated biphenyls (PCB), organochlorine pesticides and polybrominated diphenyl ethers in aquafeeds compounded using traditional FM. Maule *et al*. [167] examined feed samples from 11 US salmonid hatcheries and reported that all 46 samples, collected over a 2-year period, were contaminated by PCBs and 18 heavy metals. Comparable observations for PCBs were also noted by [168] who described a 50% contamination rate of commercial aquafeeds for various pesticides while [169,170] reported on PCB accumulations in trout tissues following feeding of laced diets. Doğu *et al*. [171] observed the effect of the organophosphate pesticide chlorpyrifos (CFP; 0.02–0.04 mg L^{-1}) on caged trout and found a significant correlation of increased CFP doses, leukocyte levels, total oxidant status, oxidative stress

index, and DNA damage, while [172] detected residues of nine different organochlorine pesticides in trout derived from Spanish farms. The wide-ranging effects of POPs in fish include disruption of the endocrine system, with negative influences on metabolism, growth, reproduction and early development, dysfunction of the immune system, and advance of neoplasia in the liver and elsewhere [review: 173]. Others caution over the possibilities for bioaccumulation of heavy metals in farmed fish fed contaminated feeds and there is a positive correlation between certain metals in feed and trout tissue accumulation [*e.g.*, 174,175]. The most important heavy metals are Pb, Hg, As and Cd and their various effects on general fish health and well-being are reviewed elsewhere [*e.g.*, 176–180].

Most recent has been the contention that FM is laced by microplastics (MP; [181–185]) and in rainbow trout it has been demonstrated that ingestion of feeds contaminated with polyethylene (PE; 46–548 μm) results in particle translocation to various tissues likely *via* persorptive pathways [186–188] with consequential negative impacts on weight gain, FCR and SGR, oxidative stress, DNA damage and apoptosis in the brain and muscle tissues and dysfunction in the expression of multiple hepatic genes [189,190]. Other studies, with varied species, reviewed by [191], also speak of the negative effects of microplastics on gut integrity, the intestinal microbiome, digestive enzyme activities and gene expression. While [191] did not observe any noticeable differences in trout growth when fed PE contaminated pellets, they did report changes in hematological and biochemical profiles, intestinal morphology and gut mucus production, and recorded inflammation of the gills, hepatic and renal tissues, and changes in gene expression for the liver and head kidney. Clark *et al*. [192] determined that ingested dietary polystyrene (PS) MP (~200 nm) found its way into the liver, gallbladder, kidney and gill tissues but was undetectable in organs and carcasses after a 7-day depuration, indicating efficient excretion. *In vitro* examination of trout immune cells revealed that polystyrene MP exposure resulted in a detrimental effect on B-cells, decreasing the abundance of non-phagocytic cells which, the authors speculated, might have a negative impact on IgM/IgT responses to pathogens *in vivo* [193]. On the other hand, ingestion of PS MP by trout has also been reported to be without effect on intestinal transport, immune function, or inflammation [194], findings supported by [195], who were unable to detect the translocation of MP (10–300 μm) into liver, gonads or fillets following 2 weeks of feeding ~9,800 microspheres per gram of food.

It is noteworthy that when in the presence of MPs, some chemicals, such as POPs, and even pathogens, can be adsorbed and, in certain instances, this may influence their accumulation and toxicity [196]. For example, co-exposure of dietary MPs with *Yersinia ruckeri* decreased catalase and glutathione peroxidase activities, and total antioxidant levels in juvenile trout, leading to the potential for increased *Yersinia* pathogenicity [197]. Co-delivery of the antibiotic enrofloxacin with dietary MPs to juvenile trout led to significant changes in hepatic superoxide dismutase (SOD), glutathione peroxidase (GPO), glucose-6-phosphate dehydrogenase and total antioxidant capacity suggesting increased toxicity of the antibiotic [198]. Using phenanthrene as a model polycyclic aromatic hydrocarbon (PAH), [199] reported elevated uptake in the presence of PS MPs in trout juveniles. Similarly, when PS MPs and the organophosphate insecticide chlorpyrifos were combined, damage to intestinal folds and gills ensued [200], while alterations in trout fillet AA and fatty acid (FA) composition, and protein content occurred, leading to decreased nutritional value of the fillet [201]. Other studies indicate that MPs weaken the accumulation and toxicity of fungicides in a particle size-dependent manner the smaller the more potent the effect [202]. MPs, therefore, function as vectors for a wide range of potentially damaging contaminants giving them a reputation as “Trojan horses”. However, this moniker may be erroneous, being appropriate only to specific sizes and types of MPs

and for specific adsorptive chemicals since [203], along with others, have demonstrated the importance of the surrounding medium to chemical uptake either with or without MPs *versus* absorption from the diet.

While incorporation of PP in aquafeeds removes some of the risks associated with FM and environmental contaminants, such as PCBs, they increase threats of spoilation due to the presence of toxigenic fungi and mycotoxins [204,205]. Toxigenic fungi contaminate crops during cultivation or post-harvest, during storage. Mycotoxins are of global concern in cereal crops used for animal feeds [206], and various studies have verified their contamination of aquafeeds [207–209], including those for rainbow trout [204,209,210]. Contaminating toxigenic fungi can reduce the nutritional value of ingredients and result in mycotoxicosis in fish, causing reduced growth, immune dysfunction, occurrence of carcinomas and even death [207,208].

A pressing issue, that has rarely been evaluated for fish, is the possible transfer of food allergens from aquafeeds (*e.g.*, peanut, glutens, etc.) to fish flesh and thence to hypersensitive individuals who, while becoming more numerous, presently represent 10% of the global population [211]. The potential for such occurrences is exemplified by [212] who established that feeding zebrafish, *Danio rerio*, FM spiked with the herring worm, *Anisakis simplex*, expressed the nematode's proteins in their flesh following 2 weeks of feeding. Upon ingestion of the zebrafish flesh, the meat initiated an allergic response in a sensitized consumer. Similar observations were made with chickens reared using FM-based feeds [213]. Allergic symptoms have also been reported following ingestion of mealworm [214,215]. However, when chicken meat and eggs were examined for the transfer of peanut and soy proteins from feed they were not detected by ELISA or immunoblotting methods [216,217]. In another study, however, Tomczak *et al*. [218], in using soy and lupine in chicken feed, reported modified egg protein composition, with those of a molecular weight of ~13 kDa potentially increasing immunoreactivity to children allergic to soy. Many allergenic proteins are heat and protease-resistant [219] which increases the prospect of reaching the intestine substantially intact and being absorbed into the bloodstream either as IgE-immune complexes, as has been demonstrated for soybean allergens with piglets [220], or in native or substantially undegraded form. Similar responses might be expected for rainbow trout which have proven capable of intact protein absorption [221,222]. Clearly though, this area represents fertile ground for future research consideration. The potential negative impact of phytoestrogens is considered under the section dealing with reproduction.

3.3. Ingredient costs

Life-cycle assessment of rainbow trout operations has determined that feed production accounts for more than 40% of the energy budget [223–225]. Not surprisingly, feed is the largest operating cost of trout aquaculture and, as the price of FM strengthens due to competition and scarcity, it is only natural that the aquafeed industry has examined the potential of plant and SCP sources as ingredient alternatives. Production of optimal diets for trout must assume that there is no ideal feed formulation, but requirements for specific nutrients that can be supplied variously without being detrimental to animal growth, welfare, or the environment, while also sustaining consumer expectations [79,226–228]. For feed manufacturers, profitability represents a key driver and they must source ingredients that meet the nutritional requirements of the animal while being competitive in the international market and exhibiting those characteristics outlined previously. Indeed, contemporary processing technologies have reduced plant ANFs, increased protein content,

enhanced palatability and digestibility, and lengthened product shelf lives, but these values come at a cost, such as addition of EAAs and PP concentrates and isolates, when complete removal of FM is the ambition (Figure 1).

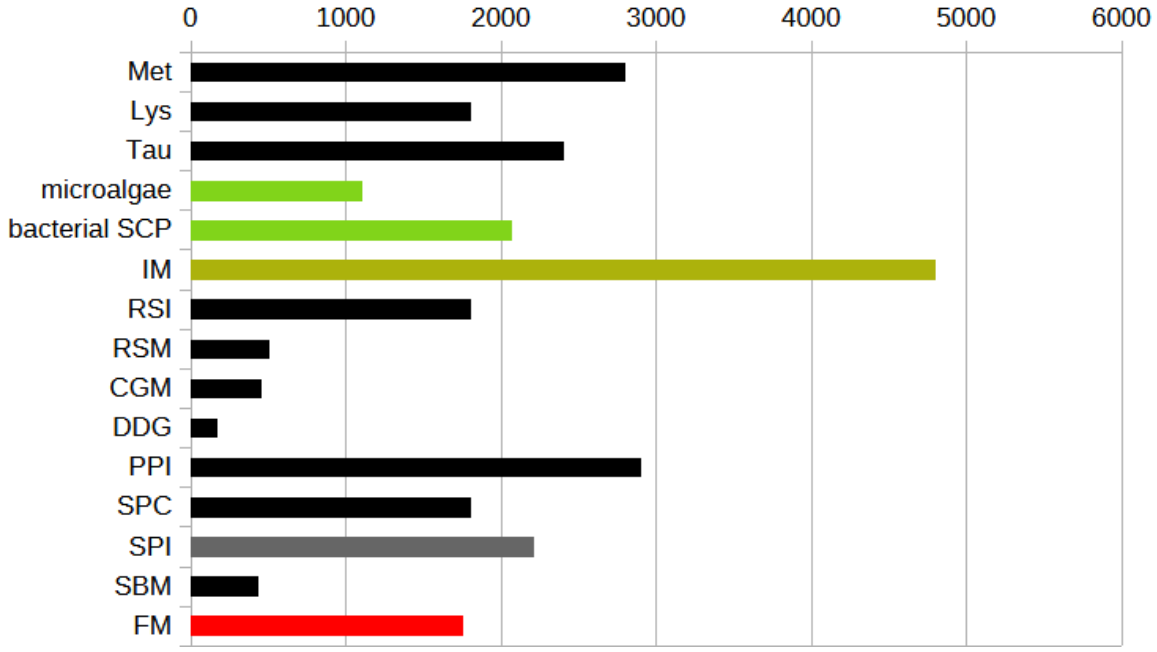

Figure 1. Average prices per tonne (US$) for FM, alternative proteins, including insect meals (IM) and AAs. Sources: [238–241].

Nevertheless, partial substitutions of FM, using 50-60% of total protein as PP, reduced the cost of producing 1 kg of trout by 25–50% when compared to a commercial fish feed [229]. Half of the PP-based formulations evaluated by [230] were reported to be ≥ \$6.69 ton^{-1} less expensive than a commercial feed when taking account of ingredients, but costs per unit gain were ≥ \$11 ton^{-1}. Lower ingredient prices were determined for peanut meal and SBM-based diets *versus* commercial feed, but higher expenses were seen for diets containing SPCs and soy flour. When evaluating the cost of ingredients per ton gain, however, the commercial feed was more efficient than all alternative protein diets. This disparity was related to the costs incurred for the incorporation of various absent (*e.g.*, EAAs) nutrients, and utilization of costly protein isolates and concentrates (Figure 1). Noteworthy, however, is that the commercial feed had higher protein (~25%) and fat (~36%) contents than the PP diets which likely influenced weight gain, FCRs and feed intake. Still, favorable growth was attained by fish fed the PP-based diets. Production costs of trout using a mixed PP feed, comprising SBM, LSM, CGM and PPC, *versus* an experimental FM-basedfeed were 4% less kg^{-1} fish produced, but 17% more when compared to trout reared on an industry manufactured diet [229] which would be expected, given industry's bulk purchasing power. In evaluating different rearing temperatures and dietary formulations [231] determined that the most cost-effective method for producing rainbow trout was with the use of a 40% PP/10% lipid feed at 14 ºC because fish attained similar weight gain to trout fed more costly (FM-based) diets. Superior responses to PP-based diets in terms of growth have been reported for strains of trout developed to accept plant feeds and these lines will likely have a positive impact upon the economics of commissioning alternative proteins. Ultimately, however,

the use of PP and SCP sources in aquafeeds will place increased pressure on agricultural production with expanded demand for land and water resources. As concluded by Pahlow and colleagues [232], future feed formulation research should consider this aspect of feed production to reduce aquaculture's terrestrial water footprint.

3.4. Growth and survival

Tables 2 and 3 summarize the results of studies that report the impact of complete replacement of FM with PPs and SCPs, respectively, on rainbow trout growth. These trials averaged 101 days in length, using fish of mean weight 58.6 g at temperatures of around 14.5 ºC. The alternative PPs employed included meals, concentrates and extracts derived from barley, canola (rape), corn, cotton, lupin, pea, peanut, potato, soybean and wheat, supplemented, or not, with various EAAs and non-essential AAs. SCPs employed included various algae, yeasts, and bacterial proteins. With some notable exceptions, most FM-free diets resulted in significantly decreased growth rates conjoined with lower specific growth rates (SGR), reduced feed intake (FI), and feed and protein efficiencies (PE). These conclusions generally match the findings of a meta-analysis on the use of PP meals and concentrates in salmonid diets at varying levels of inclusion [45]. Linked to these inferior responses *versus* FM-based diets, trout fed PP-based feeds returned increased feed conversion ratios (FCR) which, from a practical standpoint, might result in decreased profit per unit of biomass produced. However, cost differences in raw materials (FM vs. PP; Figure 1) could feasibly moderate such an outcome. Noteworthy is that survival was similar between fish fed FM- and PP-based foods. Remarkable was the lack of effect of PP-based diets on trout growth in trials extending over 180 days [*e.g.*, 231,233,234]. Some have seen better survival of trout maintained on PP-based feed [235], while others generally report parity following complete replacement of FM by PP and SCP. Seldomly, higher levels of mortality have been recorded, although these are usually, but not exclusively, from earlier studies [*e.g.*, 236,237].

Although often reported, the slower growth seen in trout fed PP-based feeds is not always a consequence of reduced FI. In some trials, where FI was the same for both FM- and PP-centered diets, protein efficiency ratio (PER) was reduced for the alternate protein-based feeds, indicating poorer use of dietary N [242,243]. This decrease in PER may emerge for several reasons including the presence of enzyme inhibitors and other ANFs (Table 1), or disruption in the net absorption profiles of individual EAA. Insufficiencies in EAA may manifest in numerous ways including loss of appetite, poorer growth and FCE, increased occurrence of cataracts, fin erosion, scoliosis, and mortality. Replacement of FM with bacterial SCPs over a 132-d period also disturbed the efficiency of amino acid absorption by trout, with perhaps only 55% of that consumed being digested. In this instance, the poor response of the animals, growth-wise, was thought to result due to deficiencies in methionine (Met), Phe and threonine (Thr; [242]); even though test diets were balanced with respect to the known EAA requirements of trout. Attempts have thus been made to eliminate the impact of these risks with the addition of limiting EAAs to alternate protein diets. When feeding non-FM-based diets it is essential that an optimal balance of AA is achieved to reach peak production efficiencies. However, because the AA profiles of many alternative PP and SCP are imbalanced some diets are over-formulated, employing surplus dietary protein to meet AA requirements [3]. In such cases, the addition of crystalline AAs can be used to spare protein and this has been illustrated by [244] who reported on the effects of supplemental glycine (Gly), Met, Lys, and Thr, in PP diets to levels equal

to that found in 450 g kg^{-1} trout muscle protein and observed improvement in weight gain and muscle ratio and a decrease in intraperitoneal fat ratios, indicating the potential for reducing protein content of PP-based diets by 4.5%. Significant advantages were seen in growth, food conversion and muscle ratios, whole body crude protein and intraperitoneal fat presence, favoring the supplemented diets. Provision should be included with such strategies, however, since crystalline AAs are absorbed more rapidly, resulting in AA imbalance even when the diet meets requirements, perhaps resulting from a flooding of AA transporters. A consequence of this can be inferior growth caused by antagonisms and alterations in their availability at sites of protein synthesis [245–247]. This scenario may potentially be avoided using multiple feedings.

Snyder *et al.* [248] supplemented a SPI trout feed with EAAs to mimic the profile of FM and this resulted in correction of differences in FCR and PRE. Yamamoto *et al*. [249] added either Met/Lys or all EAAs to a fSBM-based diet in attempts to gain growth equivalence to trout reared on a FM diet. The composition of the +EAA diet corresponded to or, for Leu, Thr and Phe, was better than the FM diet. Although these strategies increased weight gain above that of fish fed an fSBM diet alone, fish fed diets with added EAAs did not attain the weight growth of the FM fed trout. Moreover, analysis of whole-body AA composition found that fish fed fSBM + all EAAs expressed muscle AA levels that generally exceeded those of the FM fish suggesting that AA utilization was improved with EAA addition. Hang *et al.* [250] expanded on the work of [249] by examining the effect of Met/Lys supplemented fSBM-based diets, with or without bile acid, on digestive function, fillet quality and liver health. They reported that fillet Met, Cys, Tyr and Pro increased in fSBM-based fed fish compared to FM treated trout and took this to indicate that supplemental AAs augmented protein anabolism.

Trout can synthesize the conditionally indispensable aminosulfonic acid taurine (Tau) from Cys and Met via cysteinesulfinic acid decarboxylase [251,252]. However, since Tau is absent in plant proteins [253] which may also be limited in Cys and Met [254], they may be incapable of synthesizing enough Tau to meet physiological needs and to maximize growth when fed PP-based diets. Bearing this in mind, Gaylord *et al*. [255] examined whether supplementary Tau improved the performance of trout fed plant-based diets. They reported that Tau supplementation of FM-based diets was without effect on growth but, when added to PP-based food, at 5 g kg^{-1} diet, growth equivalent to that seen in FM-fed animals was reached. The latter finding, however, contrasted to the observations of [256] who recorded no benefit of supplemental Tau in fingerling trout fed a diet comprising added EAAs, CGM, SBM and WF. Gaylord *et al*. [257] also examined the benefits of supplementing PP diets with Tau (5-10 g kg^{-1}) in the presence or absence of Met over 84-d. Tau decreased circulating IGF-I but didn't impact GH and had an additive effect on growth ($P < 0.009$) and muscle ratio ($P < 0.002$) when synthetic Met was absent from the formulations, and was considered critical to gain growth and FCRs equivalent to that of trout fed FM-based diets. Similarly, Hernández *et al.* [258] examined addition of different ratios of Tau/Met to fingerlings (0.54 g) fed on diets with SPI and *Spirulina*. They reported a trend for increased weight gain and SGR with increasing levels of Tau up to 75% Tau/25% Met. Diets void of supplemental Met, however, had lowest weight gain.

Many other publications have reported on the growth of trout fed diets comprising PP and SCP that have chosen not to include a FM-based control or commercial feed for comparisons [*e.g.*, 259–263]. These reports are nonetheless of great importance, not only because they confirm that rainbow trout at all life stages are able to accept and grow on FM-free feeds but also because they provide

additional information of value relating to the production of vegan fish. It is relevant to highlight the importance of trial length in studies designed to examine the effectiveness of PP and SCP as alternatives to FM. As is clear from the tabulated studies (Tables 2 and 3), designated timeframes appear arbitrary (28-d to 2-yr) and as cautioned by [264] and others [*e.g*., 265,266], this only represents a hazard when attempting to draw conclusions about the response of trout to experimental diets. For example, de Francesco *et al*. [267] didn't observe differences between trout fed PP- and FM-based diets until after 84 days. Overturf and Gaylord [268] reported lower growth rates for a PP-based diet through 76 days but, equivalence thereafter to 167 d. In contrast, Barnes *et al.* [269] using a fSBM diet, found no differences in weight, length, SGR, or fillet composition after 94-d when compared against fish reared using FM feeds but, 205-d post-trial start, differences ($P < 0.05$) in each parameter were apparent, favoring the FM-fed group. Importantly, health indices for fin and pseudobranch condition and HSI in Barnes and colleague's study [269] were also poorer in the alternate protein group at day 205. A notable observation related to the morphological features of the distal intestine where, at 94 days post-trial start, fSBM fed fish exhibited *lamina propria* thickening and increased amount of connective tissue, which was reverted by day 205. It is noteworthy, however, that the fish fed the PP diet did not achieve the recommended [270] 300% weight gain at 94 days. Indeed, a 300% weight gain for larger fish may not be an appropriate requirement. For younger, faster-growing fish (~first feeding) a 1000% weight gain is recommended.

3.5. Morphological changes

PPs possess a wide range of ANFs (Table 1) that have negative impacts on growth and immunity. For instance, when soybean proteins are used at elevated levels in salmonid diets, the distal intestine becomes inflamed and expresses structural alterations [273]. These changes can be slight, moderate, or severe depending on the alternative protein used, its level of dietary incorporation, the degree and type of processing the ingredient receives and even with the length of study (*vide supra*). In rainbow trout, PP-based feeds have been associated with structural damage to the gills, stomach, liver, pancreatic tissue, kidney and spleen. Some strains of trout appear less sensitive to PP than others [*e.g*., 272] especially when selected for an enhanced ability to accept PPs [273]. PP diets have been associated with an elongation ($P < 0.03$) of the cardia part of the stomach [274], an increase in relative intestinal length [125] and, with a feed incorporating 20% SCP, thickening of the foregut [275]. Such architectural adjustments may act to increase the size of ingested rations, slow passage of ingested food and, or increase time for enzyme-substrate interaction and nutrient absorption. Richard *et al.* [276] reported that inclusion of protein-rich yeast at 10% and 15% of a PP diet induced changes in the internal perimeter of the trout distal gut and its ratio with the external perimeter ($P < 0.03$). They speculated that the increased diameter might have provided a greater intestinal surface for absorption. They further ventured this modification may have contributed to the enhanced growth seen. Also, the 15% yeast inclusion rate returned to a lower ($P < 0.002$) inflammation score.

Commonly with soybean and other plant-based diets, a decreased intestinal fold height and width, and an increased thickness of the *lamina propria* is recorded [*e.g*., 277–279], possibly due to the presence of saponins and other ANFs (Table 1). Nonetheless, contrary findings have been presented for both non-selected and selected fish. For example, Venold *et al.* [280] reported trout fed SBM-based feeds exhibited increased mucosal fold height and fusions, and greater *lamina propria*

width and cellularity ($P < 0.015$) in selected when compared to non-selected fish. Toledo-Solís *et al*. [281] observed differences only in the number of intestinal fold fusions, which were greater in fish fed PP-based diets, while [282] and [283], using algal meal to replace FM, found no pathological differences in gut architecture but, like [274], using pea protein isolate (PePI), recorded increased height of the intestinal epithelium and folds. Others still have observed no significant effects of PP diets on intestinal fold height, epithelium length, or stroma proportion in either proximal or distal intestine [*e.g*., 230,284,285–287], prompting the suggestion that trout may be insensitive to certain dietary PPs, express only transient enteritis [269] or differ in response with strain.

Cellularly, SPC-based feed has been shown to lower both height and width of enterocytes in the trout distal, but not proximal intestine [285], although [288] reported an increased enterocyte area and numbers of mucus cells per fold. Conversely, SBM-based feeds have been associated with general disturbance to the intestinal mucosal epithelium with increased cell sloughing [289] and a reduction of goblet cells along the gut's length [290]. Indeed, the latter two phenomena may be associated with decreased mucus reducing physical protection of the mucosa resulting in intensified mucosal abrasion. A possible consequence of decreased physical protection is an environment more conducive to cell abrasion and this may partly explain Matsunari *et al*.'s [291] observation of surface epithelial breaches. Others have commented on the partial degeneration of the absorptive enterocyte's microvillus brush border [*e.g*., 249,281,292,293] associated with changes in the apical cytoplasm. The severity of these abnormalities, which include lack of pinocytotic vesicles, presence of smaller, more numerous supranuclear vacuoles, and more apical positioning of the nucleus [273,285,289] vary with dietary ingredients and appeared less severe when fSBM replaced or reduced the content of SBM. Others have observed SCP-based diets to increase the size and number of supranuclear vacuoles in the posterior and anterior gut without disruption to gastric or anterior intestinal cell architectures [275,287].

While a wide variety of PP-based formulations have been reported to be without effect on the histology of other tissues of rainbow trout, including the spleen, gills, liver and kidney [230,283,284,286], PP-based diets appear to express hints associated with pantothenic acid (vitamin B_5) deficiency, including typical signs of nutritional gill disease – fusion of lamellae and clubbing [294]. More commonly reported are impacts of PP-based diets on values for hepatosomatic indices (HSI) and, although inconsistent, decreased HSIs have generally been reported [249,267,285,286,295–298], although no change [*e.g.*, 257,272,299–302] and augmentation [287] have been described. These differences are more likely reflective of dietary lipid composition or may illustrate differences in feed quality among studies. Generally speaking, increased visceral organ weights are positively correlated with increased metabolic activity and hence energy use and decreased muscle deposition.

Iwashita *et al*. [303], using SPC, [283] using algal meal, [295] using a fSBM, [274] using PePI, and [287] using a SCP, did not observe any severe histological change to the liver other than some increased fatty deposits. In contrast, [293] and [303] observed abnormal morphology in the liver of trout fed SBM, including atrophy of hepatocytes, obscured nuclei, lack of vacuoles and expanded sinusoids. The severity of these abnormalities, however, were reduced when fSBM was employed leading to the suggestion that fermentation of SBM and the associated reduction in soybean protein size may have had a dampening effect on the activities of ANFs. Nonetheless, in trout fed fSBM diets, hepatocytes expressed reduced nuclear and cytoplasmic diameters and lacked vacuoles [304] although this was reversed in fish that received diets supplemented with EAA [291]. Like the results achieved using fSBM, complete replacement of FM with SPC decreased ($P < 0.001$) hepatocyte

volume, without change in hepatocyte nuclei diameter [285]. In fish fed SPC, gall bladder somatic index (GBSI), and concentrations of the bile salts, cholyltaurine and chenodeoxy- cholyltaurine increased ($P < 0.05$; [303]); whereas, with fSBM and SBM, GBSI and cholyltaurine, chenodeoxy cholyltaurine and total bile acid concentrations declined relative to FM controls [249,291,293]. Substitution of FM by PePI had no effect on the bile duct ($P < 0.03$; [274]), but significant lesioning and focal areas of leucocytic inflammation of the bile duct were observed in the livers of trout fed PP-based diets void of P, Mg, NaCl, and inositol [305].

Clearly PP, and to a lesser extent SCP diets, have the potential to perturb the normal architecture and hence functioning of trout tissues, be this temporarily or over the longer term. Either way, such disturbances will interrupt normal homeostasis and conceivably provoke modification to various systems (neuroendocrine, immune) and thereby have an energy-demanding and growth-depleting corollary. Negative consequences of alternate proteins, especially to the patency of the gut and gills are of heightened concern because they represent major portals for microbes and hence infection. It does appear that more serious and wide-ranging tissue modifications are particularly associated with the use of soybean and its derivatives. Given that these products potentially contain antiprotease, phytohemagglutinin, lectin, glucosinolate and saponin activities, this perhaps shouldn't be too surprising although pea and potato proteins are similarly endowed with a variety of ANFs (Table 1). Judicious use of a broader diversity of alternative proteins in trout feeds might temper the potential for negative structural effects.

3.6. Gastrointestinal transit

Borey *et al*. [410] investigated gastric filling and intestinal transit rates in trout fed a FM or PP diet after a 72-h period of starvation. The authors then sampled fish using a subjective scoring system to assess degree of fullness (empty, partially filled and full). Prior to starting the experiment, the stomach and intestine of sacrificial fish were checked to ensure 100% emptiness (time 0) then, at 2, 6 and 12-h post-feeding, animals ($n = 9$ per diet and sampling point) were sacrificed, dissected and, following lateral incisions of the pyloric stomach and intestine, undigested food, chyme, partly and fully digested material were removed. Two hours post-feeding around 90% of animals, irrespective of diet, expressed gastric fullness and even at 12-h the stomach of most trout assessed remained full. Indeed, earlier studies [411] indicated a 36-h time-course for complete evacuation of the stomach, which was influenced by meal size and type, with larger meals being evacuated more rapidly. In Borey *et al*.'s [410] study, the anterior intestine was partly filled at 2-h for >55% of all fish and, at 6-h, 70% of PP-fed animals had partially filled intestines with ~10% being fully extended. In contrast, at 6-h post-feeding the FM diet, trout only exhibited ~40% of individuals being full and 55% being partially full and, at 12-h post-feeding, this treatment group expressed >60% full; whereas, the intestine of ~40% of PP-fed fish were deemed full [410].

The control of food intake and gastrointestinal function, including secretion events and transit times, is thought to be regulated by nutrient sensing systems associated with several hypothalamic, intestinal, and hepatic peptides [*e.g*., 412–415]. In trout, AA sensing systems located in the hypothalamus and telencephalon are believed to control food intake [414] which seems to be justified given [416] demonstrated that intracerebroventricular infusion of leucine (Leu) reduced food intake, while valine (Val) increased ingestion, together with the appropriate expression of hypothalamic and telenchephalic neuropeptides. Further support for the existence of active nutrient

sensing systems in trout has been provided by [417] who used intragastric infusions of Leu and Val, together with proline (Pro) and glutamate (Glu), to illustrate modulation of gastric and intestinal calcium-sensing, taste, and G protein-coupled receptors. Other studies, including the work of [340] who examined olfactory sensing in trout supplied from first feeding with a PP diet, record increased expression of *ora5b* (olfactory receptor), *calb2a* (calcium binding) and *gnaolf1b* (a G protein signaling gene) in the olfactory rosette when compared to fish fed a control diet. The results of [355,356] with alevins likewise indicate disruption in early feeding responses with PP-based feeds.

In trout, several hormones are intimately involved in the normal functioning of the GIT. For example, gastrin which is released following gastric distension and presence of protein-rich food, influences gastric motility and hydrochloric acid secretion. Glucagon-like peptide-1 (GLP-1) is secreted in response to FI and works to decrease gastric emptying thereby taking on the role of a satiety factor [418]. Cholecystokinin (CCK) triggers bile and enzyme release into the intestine from the gall bladder and pancreas and, like GLP-1, plays a role in inhibiting gastric emptying in trout. When a PP selected line of trout was fed an EAA supplemented PP diet, gene expression for CCK levels were increased [419] indicating slower stomach voidance and prolonged protein digestion and absorption. Serotonin has also been associated with controlling appetite through neuropeptidal regulation [420]. Clearly, evidence points to a rather complex interconnected system of feed intake and sensing in trout that is deserved of further examination. For example, more precise methods of quantifying fullness and emptying of the stomach and intestine are needed to evaluate the impacts of different dietary formulations on digestion and absorption. It has been suggested that gut evacuation rates of PP-based feeds can be protracted, and this increased retention time may facilitate augmented microorganism-enzyme-substrate interactions to occur within the gut lumen and assist in the digestion and absorptive process and this requires more study. As comprehensively considered by [421] converting carnivores into vegans has wide-ranging consequences for their performance and while great strides in our knowledge have been made over the last 30 or so years, especially with salmonids, our comprehension of the many impacts PP have on intestinal physiology and function remain imperfect.

3.7. The gastrointestinal microbiome

The trout gut harbors both residential (autochthonous) and ephemeral (allochthonous) species of viruses, bacteria, archaea, and eukaryotic microbes. Together these organisms form the gastrointestinal microbiome. Autochthonous microbes colonize the host's epithelial surface; whereas, allochthonous species, under normal circumstances, are transient, being expelled in fecal material [422,423]. The natural gut microbiome plays critical roles in modulating nutrient absorption, and in synthesizing enzymes, vitamins, AAs and SCFAs. It is also engaged in intestinal development, providing energy for epithelial cells and essential work in immunomodulation and maintenance of gut barrier function**.** Net. Many studies have revealed the complexity of the trout gut's autochthonous microbial community and have established some of the principles governing the assembly and preservation of its core membership [*e.g.*, 284,424–435]. The core microbiome refers to groups of specific microbial taxa or genes that are widespread in the gut and have functional significance in maintaining host homeostasis. Microbial colonization of the fish gut is influenced by organisms present on eggs and in the surrounding environment. Following first feeding, considerable diversification in the gut's microbial community ensues, and extensive changes in the microbiome occur as the animal grows older. Species-distinct microbial communities establish in different segments

of the intestine, with a proximal to distal gradient, progressively increasing in density [422,423]. Since variations in the structure of the gut core microbiome differ between freshwater and marine species, it is likely that such differences are discernible in anadromous steelhead (*Oncorhynchus mykiss irideus*) and redband (*O. m. gairdneri*) trout following transition from freshwater to the marine and *vice versa*.

Perturbations in the trout gut's core microbial community are known to occur due to dietary manipulation [*e.g.*, 358,372,436–451; Figure 2A and 2B], and feeding PP-based diets has been reported to impact gut microbial diversity and the proportional abundance of specific phyla (Figure 2A and 2B). For example, the relative abundance and ratios of *Firmicutes*, *Proteobacteria*, *Bacteroidetes* and *Actinobacteria*, the most prominent gut bacterial phyla, change with higher levels and different types of dietary PP [*e.g.*, 315,372,429,438,439; Figure 2A). These adjustments are associated with a richer diversity of microbes, parodying the microbial community assemblages of the gut of fish from lower trophic planes [423]. In fish fed PP-based diets bacteria predominate in the mid- and hindgut whereas in fish fed animal protein, eukaryote presence increases [315].

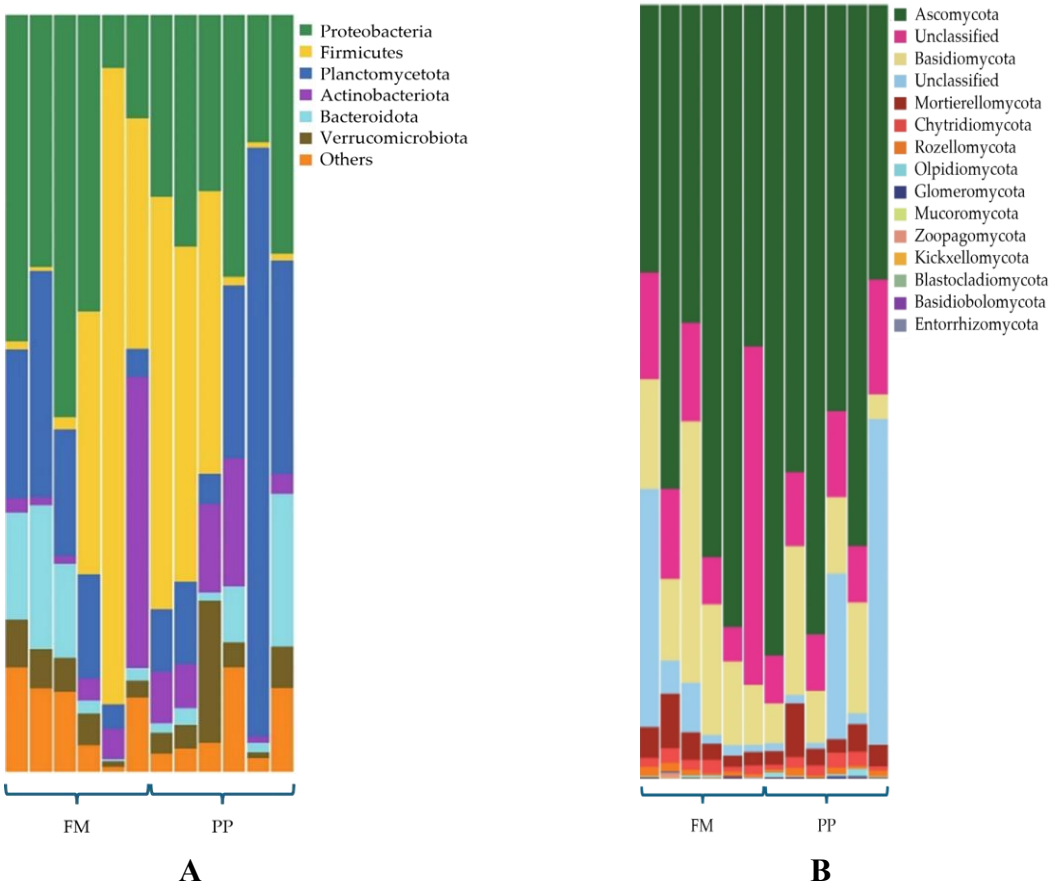

Figure 2. Relative abundance, in percent, of core gut microbiota from six individual rainbow trout fed either a FM- or PP-based diet. A) bacterial phyla, B) fungal phyla. The figure illustrates the distinctness and variability of microbiota in animals that have been reared under identical conditions of husbandry. Image presentation form, but not dataset, is slightly modified from [390], and is used under the article's Creative Commons License (http://creativecommons.org/licences/by/4.0).

Table 3. Responses of rainbow trout to diets in which FM has been completely replaced by single-cell proteins.

Start size, days, temp	Main proteins examined*	Response of fish relative to FM-based feeds	Ref.
15.7 g, 128 d, 15 °C	Y, SBM, SPC, CP	↓ growth and FI ($P < 0008$)	[392]
91.3 g, 88 d, 15°C	SBM, CPC, SPC, DDG, BPC, *Spirulina*	No wt differences, FCR and SGR varying with dietary inclusions of PP ($P < 0.001$)	[393]
2.22-2.67 g 32 d, 17°C	*Geotrichum candidum*	↓ growth ($P < 0.05$)	[394]
150 g, 56 d, 15 °C	WM, WG, SPC, *Arthrospira Platensis*	No diff. wt gain, SGR, FCR, PER	[395]
44 g, 84 d, 17°C	*Torula* Y, CGM, SBM, FBPC, PePC	↓ growth ($P < 0.05$) bar 10% Y group ↓ SGR in plant-based feeds	[396]
70 d		↓ growth, SGR and *K* ($P < 0.05$) ↑ FCR ($P < 0.05$)	[397]
6.8 g, 50 d, 14°C	SBM, *Spirulina* powder 50:50	↓ growth, SGR ($P < 0.05$) ↑ FI, FCR ($P < 0.05$)	[398]
16.7 g, 56 d, 15-18°C	Enzyme-treated SBM, CGM, Y, BMA	↓ growth, SGR, survival, *K* ($P < 0.05$) ↑ FCR ($P < 0.05$) Addition of EAA and bile acid resulted in growth equivalence to controls, but with poorer survival and FCR ($P < 0.05$)	[250]
0.54 g, 70 d, 13 °C	SPI, *Spirulina*, WG, + 0.5% Tau/Met	↑ growth, SGR ($P < 0.05$)	[399]
68 g, 56 d, 14°C	*Chlorella*, CPC, SPC, WGM, WF	↑ wt gain, SGR ($P < 0.05$)	[282]
275-393 g, 28 d, 12-17 °C	Bacterial protein Algal protein, Y	↓ wt gain, FI ($P < 0.05$) for all diets	[400]
19.1 g, 30 d, 10°C	Cas, SCP	↓ wt gain ($P < 0.05$)	[401]
7.6 g, 60 d, 14-17°C	Neogreen algae	↑ wt gain ($P < 0.05$)	[402]
8.5 g, 132 d, 10-15.5°C	BSCP	↑ FI, ↓ growth, SGR and nitrogen absorption ($P < 0.05$) Differences in (non)-EAA absorption efficiencies ($P < 0.05$)	[242, 403]
21.7 g, 63 d, 14±1.5 °C	SCP, RDDG, YE, WF, SBM	↓ wt gain, SGR, *K* and FI ($P < 0.001$) ↑ TGC ($P < 0.001$)	[287]
100.8 g, 70 d, 16.8°C	*Arthrospira platensis*, WGM, WM, SPC	↓ wt gain ($P < 0.05$) ↑ FCR ($P < 0.05$)	[404]
49 g, 84 d, 17°C	*Spirulina*, GDDY, PP	GDDY fed fish ↑ wt gain v. PP fed fish ($P < 0.05$) FCR ↓ in PP and GDDY v. FM cont. ($P < 0.05$)	[405]
49 g, 84 d, 17°C	*Spirulina*, WG, PeP, FBPC, SBM, SPC, RSM	No difference in wt gain or SGR ↑ FI and FCR ($P < 0.05$) ↓ *K* for 2/3 groups.	[406]
22 g, 70 d, 10.0 °C	Y, SCP	No differences in growth or feed efficiency	[407]
20 dph, 374 d, 11.3-21.6 °C	EWW, PPC, CG, WG, Y	↑ growth, TGC ($P < 0.001$) ↓ *K* ($P < 0.05$) Genetically selected for PP feeds	[408]
24 g, 60d, 16°C	SBM, SM, *Spirulina*/*Chlorella*	↑ growth, SGR ($P < 0.05$) ↓ FCR	[283]
2.5 g, 42 d	SCP	↑ FCR ($P < 0.05$)	[409]

In rainbow trout, [372] reported that replacement of dietary FM by PPs resulted in significant changes to the gut microbiome. Nevertheless, as previously determined by [284], fish expressed a core microbiota comprising *Proteobacteria*, containing six orders, one *Firmicutes* and one

Actinobacteria that was largely unaffected by diet. Rearing with the PP diet resulted in increased ($P > 0.002$) abundance of phylum *Firmicutes* but decrease ($P < 0.0002$) in *Proteobacteria* when compared against the FM diet. Five species of the order Lactobacilliales were more abundant ($P < 0.05$) in trout fed the FM-based feed. Later studies revealed changes in the mid-intestinal microbiome of fish fed a SPC/CGM/WGM-based diet for 214 days *versus* animals reared on a FM-grounded feed both at high and low densities. Irrespective of feed presented or holding density, the relative diversities and abundances of shared and core bacterial classes were similar. Core microbiotas were predominated by *Bacilli*, *Alphaproteobacteria*, and *Gammaproteobacteria*. However, examination of treatment accessory core microbiota (representing 3.7–5.3% of the treatment core) exposed modifications in diversity and relative richness of uncommon components. For example, the comparative abundance of the family *Lactobacillaceae* and its genus *Lactobacillus* were significantly enriched in trout fed the PP-based feed, regardless of rearing density, as too was *Streptococcus* spp. It has been suggested that because both the latter genera contain species that impart known probiotic effects, that they may influence the animal's physiology in terms of growth rates, perhaps through competitive exclusion of other microbes and reduction of diarrhea. On the other hand, fish reared using the FM-based diet expressed an increased abundance of the family *Clostridiales* and *Clostridia* spp.

Biasato *et al.* [316] recorded greater species richness in the microbiota of plant-fed trout, with *Lactobacillus* spp. being more abundant than that expressed by the control diet, with *Cetobacterium* and *Anhydrobacter* genera represented only in the fish fed PP diets. However, [392] and [450] found no difference in the gut's alpha diversity irrespective of SCP/PP or FM-based diet. When examining the effect of temperature (14 *versus* 18^{o}C), however, a family effect was apparent with the *Aeromonadaceae* and *Enterobacteriaceae* dominating at the higher temperature and *Mycoplasmataceae* at the lower. Zhang *et al*. [219] reported that a mixed PP meal decreased gut diversity of both bacteria and fungi and [358] reported similarly, adding that PP-based diets also decreased biodiversity with age. The reduction in microbial diversity was echoed by [447,448] who observed that high carbohydrate/reduced PP-based diets caused a decrease ($P < 0.05$) in microbial diversities in both digesta and mucosa, with *Ralstonia* dominating in digesta and *Mycoplasma* being more abundant in mucus. Zhang *et al*. [219] reported that *Mehtylobacterium* was more abundant in the gut of PP-fed fish which, they suggested, might be due to their ability to degrade carbohydrates and short-chain FAs. Fungal genera too, including *Acremonium*, *Penicillium* and *Pleosporales*, were positively associated with PP diets. Knowledge of gut microbial community structure, and how this may adjust to different conditions, while interesting, is nonetheless of little worth. Conversely, identifying the functional significance and contribution of species comprising the gut microbiome provides a blueprint by which host health and performance may be beneficially influenced. High-throughput sequencing techniques, combined with "multiomic" analyses now provide the means to comprehensively explore microbial communities and dissect their functional activities [422].

Broadly speaking, the *Firmicutes* include species involved in fermentation of plant polysaccharides to short-chain FAs and others that influence FA absorption and lipid metabolism through modification of bile salt action [451]. *Proteobacteria* contribute to protein and carbohydrate metabolism within the gut and reverse oxidative damage to Cys and Met. They play a key role in preparing the gut for colonization by anaerobes and are thereby involved in regulating immunity, tight junction patency and apoptosis of enterocytes [452]. Intestinal blooms of *Proteobacteria* have been linked to inflammatory events. The associated dysbiosis may include decreased populations of *Fusobacteria* and *Firmicutes* [453]. *Bacteroidetes* possess broad metabolic potential, providing AAs

and vitamins to the host and being engaged in the degradation of complex polysaccharides, proteins and in FA metabolism [454]. They are also associated with deconjugation of bile acids and thus participate in their enterohepatic recirculation [455]. The *Actinobacteria* play a protective role in the gut since they produce immunomodifying, antibacterial, antifungal and antiviral compounds. *Actinobacteria* have also been associated with the production of growth promoters [456]. While the latter four phyla predominate the gut microbiome, that is not to say other, less populous microbes, numerically obscured from view, do not participate meaningfully in host health and physiological control processes. Understanding the mechanisms that beneficially adjust the gut microbiota, leading to enhanced FE and optimized health and growth, will certainly play a key role in attaining the goal of complete replacement of FM from aquafeeds without penalty. However, as has been pointed out previously, production-length studies are required to determine how the gut's microbiota changes with feed, age, rearing conditions, genetic background and otherwise, to develop effective management strategies to retain or beneficially modify gut ecologies, avoid dysbiosis and promote production efficiency and, as a major portal for pathogens, to investigate the effect of the gut microbiota on the physical integrity of the GI tract and its secretions [445,457–459].

3.8. Protein digestion

Following ingestion, proteins are initially processed by mechanical (peristalsis) and chemical (proteases) actions in the stomach. Thereafter, according to the classical theory of protein digestion, the resultant digesta or chyme is passed to the intestine and further degraded by luminal proteases (pancreatic endopeptidases trypsin, chymotrypsin elastases I and II and the exopeptidase carboxypeptidase A and B; [459]) to its constituent parts (AAs) prior to absorption. However, the classical theory has never been formally proposed in scientific literature. Rather, it represents an amalgamation of findings derived from a century of observations [460] and today, even though the classical theory is still taught almost as gospel it is evident that proteins are absorbed by the gut in intact, polypeptide, and peptide form and as individual AAs [461,462]. In rainbow trout, evidence for intact protein absorption is derived from ultrastructural [463–465] and biochemical [221,466] studies, while the use of a dual cannulation model, combined with hepatic portal vein blood flow measurements [467] provide net absorption data of a marker protein (horseradish peroxidase; HRP) at 0.2% of delivered doses over a 75-minute time course (Figure 3). In 24 h this may increase substantially and [466] suggested that 6% of ingested protein may be absorbed intact. Confirmation of polypeptide uptake in salmonids has been provided using bioactive releasing peptides in suppressed and enhanced models [468,469], whereas carrier-mediated H^+-dependent transport of di- and tripeptides by the trout absorptive cells has been established for a wide variety of fish species including the rainbow trout [470,471]. Importantly, peptide bound AAs are absorbed more efficiently than their constituent AAs and it is the peptide transporter 1, or PepT1, together with diffusion [472], that is responsible for the translocation of 400 peptides and 8000 dipeptides from the gut lumen. Oligo-, tri- and dipeptides that are not transported by peptide transporters are further hydrolyzed by the action of membrane-bound peptidases [473]. Resultant individual AAs from protein digestion are absorbed and translocated across the cell where they are released into the bloodstream by a separate set of transporters in the basolateral membrane [474]. Figure 4 provides a generalized overview of the mechanisms and components of the intestinal epithelium engaged in the absorption of AAs, peptides and intact proteins.

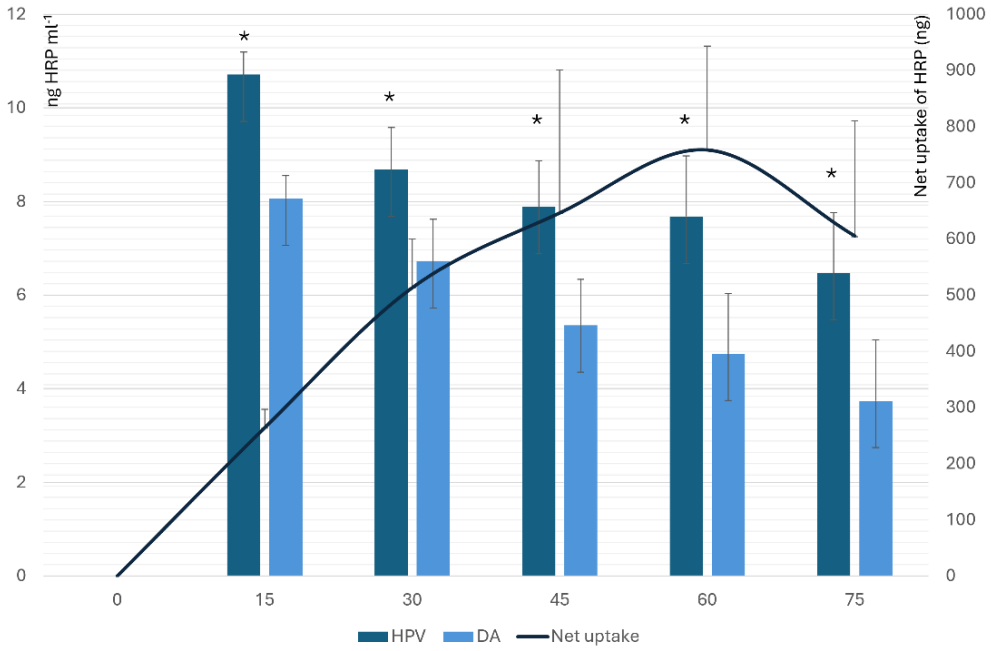

Figure 3. Net absorption (net appearance = Fpv [C_{hpv}-C_{da}], where F = portal vein blood velocity [ml min^{-1}] and C_{hpv} and C_{da} are the concentrations of the intact tracer protein HRP in the hepatic portal vein [hpv] and dorsal aorta [da] respectively) of HRP by the gut of free-swimming, 24 h fasted, dually cannulated rainbow trout (~1 kg) receiving an oral dose of 20 mg HRP kg-1 over a 75 minute timeframe. HPV concentrations of HRP were always significantly higher ($P < 0.05$, asterisks) than that measured in the DA at the same timepoint (McLean and Ash, unpublished).

Interestingly, there appears to be differences in the temporal expression of AA intestinal transporters between trout selected to accept PP diets and regular strains. Thus, PepT1 levels increased over time, and following AA supplementation with Lys, Met and Thr, *versus* control fish which the authors [475] suggested might represent a partial explanation for the improved growth seen for the selected trout. Fernández-Maestú *et al.* [330], likewise reported upregulation of mRNAs encoding for PepT1 in PP-fed trout but a decrease for the amino acid transporter Lat4. These findings have led to the proposition that AA transporters might represent useful biomarkers for future selection programs. However, PPs employed as ingredients in trout feed commonly possess antiproteases (Table 1) which interfere with protein digestion both within the stomach and intestine with consequent negative impacts on growth, body composition and metabolism. Thus, feeding trout fSBM-based diets decreased ($P < 0.05$) the activity of pepsin, gastric amylase and intestinal proteases [250].

The impact of antiproteases on luminal enzyme activities has been examined using markers of both digestive and absorptive capacity. Thus, alkaline protease, trypsin, chymotrypsin and α-amylase have been applied to evaluate digestive capability, while L-leucine aminopeptidase and alkaline phosphatase (ALP) have been deployed to assess absorptive proficiency. Examples include the work of [327] who reported a post-prandial increase of trypsin for trout fry fed a SBM extract, and reduced intestinal trypsin and leucine aminopeptidase activity (but heightened ALP activity) in trout fed a diet comprising SCP, SBM and rice-derived distillers dried grains [287]. A decrease in α-amylase (but elevated aminopeptidase N) in trout fed a mixed PP-based diet was observed by [297], and

reduced ALP in fish fed a mixed PP feed [235,476]. A 1 to 36-h post-prandial time course change in ALP activity for trout (120 g) force-fed a PP diet has been presented by [125] and, when compared to FM-fed animals, no changes were observed. Likewise, α-amylase, which expressed a peak, albeit small, 3-h post-feeding, did not differ from that of controls.

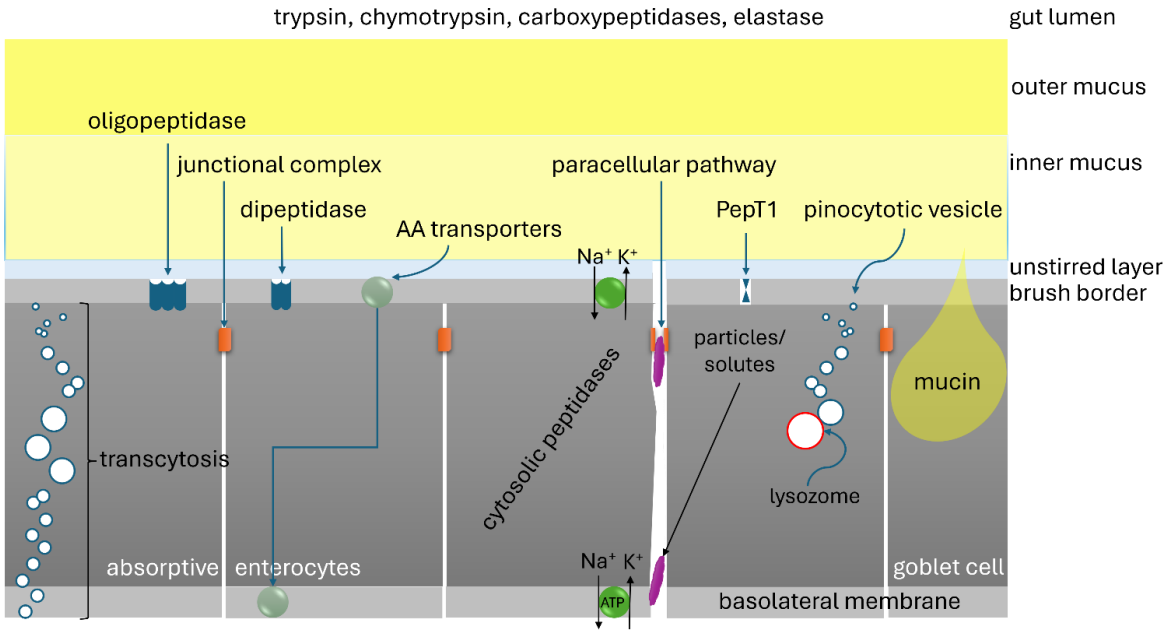

Figure 4. Simplified diagram of mechanisms involved in gastrointestinal absorption and transport of ingested protein by rainbow trout. Paracellular pathways, as illustrated by persorption, allow the passage of intact proteins/solutes and particulates between cells; whereas, transcytosis represents a transcellular mechanism for the same, which may or may not include lysosomal fusions (forming lysozomes) and thence enzymatic degradation. As their names imply, oligopeptidases and dipeptidases cleave polypeptides (< 30 AA long) and peptides (< 8 AA in length) respectively. Products of these brush border enzymes (individual AAs and smaller peptides) pass into and out of the cytoplasm of the enterocyte by an array of transport proteins. While free AAs enter the cell via Na^+-linked transporters, di- and tripeptides access the cell through the high capacity, low-affinity proton-dependent peptide transporter 1 (PepT1). Once in the cell, peptides can be further cleaved by cytosolic enzymes and metabolized. Alternatively, they may be transferred to the basolateral membrane for transport into the portal bloodstream.

Rajesh *et al*. [287] suggested that reduced intestinal trypsin and leucine aminopeptidase activities may result due to diminished nutrient digestibility of SCP cell walls and or its components. They further speculated that an observed increase in ALP activity may have been a response to bacterial lipopolysaccharide (LPS) and nucleotides because the enzyme participates in dephosphorylation of the toxic lipid moiety of LPS, blocking inflammatory responses. By binding on proteolytic enzymes, antiproteases, of which there are many thousand found in grasses, legumes, nightshades and brassicas [124], severely reduce protein digestibility and, over the longer term, may weaken the animal's production capacity, thereby reducing nutrient utilization and gut function.

Also, such intestinal dysfunction may be accompanied by a switch in enzyme production to those more resistant to prevalent antiprotease [124]. As well, the actions of antiproteases may alter the standing of the various transport systems which might feasibly result in changes in absorption profiles of AAs, peptides, polypeptides and intact proteins [126] and affect the development of intestinal inflammation.

3.9. Protein absorption

The post-prandial absorption of EAAs by rainbow trout following feeding of FM-based diets varies greatly. The reasons for this are diverse and may even reflect the fishing method employed for forage species, seasonal impacts on body composition, storage and handling characteristics of raw materials, the species (and mix) exploited for meal production, presence of by-catch and by-products, the processing methods used, feed constituents other than FM, *inter alia*. EAA appearance-clearance profiles are also influenced by experimental conditions including water quality characteristics, the age, strain and health of trout examined, the method and vessel(s) accessed for blood collection and subsequent sample manipulations and storage. The passage of time has also witnessed the use of more sophisticated methods and honed protocols for AA analysis which affects quantification, as too do column resins their care and regeneration, the standards employed and laboratory practices relating to column and standard storage. The mean 3-h post-prandial EAA profile from trout fed a crystalline EAA or whole protein diet were for the majority, higher with the crystalline AA diet, indicating more rapid absorption than protein-bound EAAs, which may increase amino acid deamination and reduce protein synthesis [477].

The first estimates of net absorption of AAs for free-swimming trout were presented by [478], 3 h post-feeding. The significance of this research is related to the potential that the model employed could have for assessing the relative replacement values and requirements of novel proteins and supplemental amino acids in trout feeds [467]. Akin to the absorption of intact proteins (Figure 3), they reported that HPV amino acid levels were always higher than concentrations measured from samples taken from the dorsal aorta. However, the timing of blood collection was too early because force-feeding a fish protein concentrate to 48-h starved 300 g rainbow trout resulted in EAA peaks 6-12 h thereafter [479] or longer [480]. Brezas and Hardy [475] reported that when force-fed a slurried diet based on SPC, HPV plasma values for Thr, Val, Ile, Leu, Met, Phe Lys and Arg, in fish selected for plant-based diets, showed peaks at 12 h ($P < 0.01$), and subsequently declined. In the caudal vein, there was an initial decline in AA levels between 3 and 6 hours followed by an increase at 12 h and a plateauing over the following 12 h. In non-selected fish, the peaks in the HPV did not occur until 18 h post-feeding while for the caudal vein, levels appeared to plateau by 3 h. When using CPC or WG-based slurry, however, ensuing AA profiles differed, both in selected and non-selected animals. Absorption profiles of AAs mimicked the composition of ingredients and maintained their ratios post-uptake [475] and similar responses were observed by others [118]. Differences in appearance-clearance profiles for EAA between artery and vein likely resulted due to strain effects. The work of Rolland and colleagues [481] is of particular interest because they examined post-prandial EAA profiles in trout fed a FM-based diet, a plant-based feed and the PP feed supplemented with limiting amino acids *viz.* Lys, Met, His, and Thr. The AA supplemented diet provided a more balanced profile of AA, as a percentage of dry matter, which was like that expressed by the FM diet, differing only by ~6.5% versus 12.6% of the unsupplemented feed. They reported that plasma profiles of EAAs

reflected dietary levels, with the feed containing the supplemental EAAs presenting faster increases and decreases in plasma levels when compared with the FM and PPC diets. Difficult to resolve from their trials, however, were declines in plasma EAAs for 3+ h following forced feeding—even for those diets supplemented with crystalline AAs. Nevertheless, the study did provide a picture of absorptive events for the dietary types examined and illustrate how supplemental EAAs may upset post-prandial appearance-clearance profiles for limiting EAAs.

The research of [311] recorded differences in concentrations of various AAs in plasma of ~240 g fish 6–8 h post-feeding three different protein sources: FM-barley, plant concentrates and plant meals. Plasma variations in EAA profiles appeared to be dependent on protein source and nutrient density; unfortunately, a restrictive timeline for blood collection was employed so that time-course events could not be followed extensively. On the other hand, Larsen *et al.* [482] studied the effects of replacing 59% FM protein with a mix of PP sources using 90 g fish. After acclimating the animals to diets, fish were starved for 48 h, re-fed and subsequently sacrificed at various time points 2–72 h thereafter, with blood being collected from the caudal vein. The authors observed that EAA illustrated peaks between 6–8 h post-feeding but, interestingly, with a 4 h delay for those fed the PP mix (i.e., peaking ~12 h post-feeding). An 8 h delay in EAA absorption from PP-based feed was recorded by [118], who suggested that the observed absorptive hesitation may have come about due to delayed gastric evacuation of the PP diet, or differences in the rates of digestion between animal and plant proteins. AA absorption in pyloric caeca through to the proximal/distal gut segments of PP fed trout was delayed which provoked [408,483] to propose that this allowed extended uptake of metabolized nutrients. The absorptive processes for EAA might also differ due to their relative contents in proffered feeds, due to starvation prior to refeeding, or method of feeding and feed type, dietary (non-protein) energy source and energy content, stress level and other factors. It is noteworthy that [394] did not observe any differences in plasma AAs between SCP and FM-based fed trout, 6 h post-feeding; whereas [387] measured increased plasma concentrations of His, Met, Val, Ile, but decreased levels of Arg and unaltered amounts of Phe and Trp in fish fed a SPI feed. Ekmay *et al*. [396] found no difference in total plasma AAs between PP (+/- torula yeast) and FM-based diets while [302] observed a higher level of circulating AAs in fish fed PP diets compared with those fed a FM feed after 48 h. They interpreted this as resulting from delayed absorption or lower AA utilization from the PP diet. This was accompanied by an early absorption of Met and Lys, accumulation of EAAs in the muscle and depletion of Glu. The authors forecasted that the imbalanced supplies of EAAs would negatively impact protein synthesis and emphasized the need to synchronize the delivery of all EAAs to muscle to ensure effective protein turnover and muscle growth. As suggested by [484], the changes observed in plasma EAA profiles in trout fed PP diets imply increased protein turnover together with a promotion in muscle protein mobilization and probable alterations in hepatic metabolism. Of high significance are the observations of [485] who noted that undernourishment of a single AA can perturb the operational efficiency of AA transporters which may have substantial consequences when using diets with a nutritionally imbalanced AA profile.

3.10. Minerals

Mineral availability and/or utilization from PPs varies with plant species, their growing environment and presence of ANFs. PP-based feeds, therefore, may be short of true requirements for certain minerals with ANFs negatively affecting the bioavailability of some, and this could lead to

sub-optimal fish performance including negative impacts on growth, development, reproductive performance and movement. In accord with the observations on growth, [486] examined the effect of adding either ash from fish protein concentrate (FPC) or dicalcium phosphate dihydrate to SBM diets at 6%. These sources were selected because SBM is a poor resource for P and Ca. He reported that fish receiving the supplements grew faster ($P < 0.01$), utilized feed more efficiently ($P < 0.025$) and expressed higher ($P < 0.01$) bone and carcass ash levels than control groups. Barrows *et al.* [305] determined that supplementation of PP-based diets with K, Mn and Na also improved trout growth, PRE and energy retention efficiency (ERE) relative to control groups, while [36] studied the effects of Mn supplementation of a mixed PP diet fed to juvenile trout for 84-d. Mn is known to impact appetite, muscle growth, movement and development. It is also an essential component of antioxidant enzymes, such as superoxide dismutase (SOD), and is engaged in FA and AA metabolism. Weight gain of treated fish in the study of [36] increased ($P < 0.05$) with optimum Mn supplementation for growth and hepatic SOD activity being determined by a non-linear model as 5 g kg^{-1}. The same authors [388] reported a need for supplemental Zn in plant-based diets when compared against FM-containing feeds, with the former requiring an additional 30 mg Zn kg^{-1} to maintain normal growth and to lessen signs of insufficiency, which were reflected in reduced whole-body Zn concentrations in the PP fed fish.

Prabhu *et al*. [373] compared the post-prandial absorption of minerals in trout fed FM and PP-based diets that had been provided with mineral supplements to cover requirements as recommended by the NRC [270]. Blood samples were taken eight times over a 24-h period and in general, for the PP-based diet, the minerals examined exhibited peak levels around 4 h post-feeding. Nonetheless, Mn, and to a lesser extent Ca, were exceptions, peaking at 30 minutes and then rapidly declining. Differences were also observed in the magnitude of mineral absorption between FM and PP diets with the former expressing increased concentration although the composition of PP-based diets did not affect mineral supply for P, Mg, K, Cu or Zn [374]. However, Dietz *et al*. [385] reported a significant reduction in whole-body Zn levels in fish fed a diet comprising undephytinized solvent-treated RSM. Plasma P levels were higher in trout fed FM-based feeds when compared against PeM, rapeseed and SPC-based meals, but plasma Ca and Mg were unchanged [487]. Zn, however, was lower ($P < 0.05$) in FM-fed animals compared against those fed the rapeseed protein concentrate-based diet. Flores *et al*. [398] reported a decrease in fecal P in trout fed 50:50 *Spirulina* powder/SBM mixed diet. Prabhu *et al*. [375] undertook a study to evaluate mineral insufficiencies in PP-based diets based on whole-body accumulations and NRC [270] recommendations. They found that a PP-based diet had inferior apparent availability coefficient (ACC %) for P, K, Fe, Cu, Mn, and Zn ($P < 0.05$) when compared to a FM diet. However, when both PP and FM diets were supplemented with 10 g kg^{-1} mineral packet, comprising Fe, 52.5 mg; Cu, 7.5 mg; Mn, 12 mg; Zn, 14 mg; and Se, 0.15 mg/kg diet, on an as-fed basis, Cu was elevated, while Mn, Se and Zn were lower in PP fed fish. The subordinate levels of Se and Zn measured were considered insufficient to meet normal body mineral levels. Similar findings were reported by [488] indicating a need for further research on mineral availabilities in feeds manufactured using alternative proteins.

Because Se is often limited in PP diets [331] examined the effect of Se supplementation on trout growth performance. As Se is a component of antioxidant enzymes, including glutathione peroxidase (GPX) and impacts flesh quality [489], they also examined lipid peroxidation and hepatic and renal GPX activity. Trout of 42 g (17.5ºC, 84-d) were held under normal and stressful conditions (↓ water flow from 24 to 12 L min^{-1}, DO_2 from 8 to 5.9 mg L^{-1} and stocking density 20 to 42 kg m^3) to

evaluate the effects of organic (Se-enriched yeast) and inorganic (sodium selenite, Na_2SeO_3) Se sources. No differences were observed in whole-body composition, growth performance, FI or FCR between control (0 Se supplementation), Se-enriched yeast and Na_2SeO_3 groups. However, stressful rearing conditions had a negative impact on FCR. Plasma levels of oxidized and reduced glutathione did not differ between dietary treatments, but both were lower in stressed environments. Muscle lipid peroxidation was lower in fish fed the Se-enriched yeast when compared against control samples while specific activity of hepatic and renal Se-dependent GPX was improved in fish fed supplemented Se. As documented earlier by the same authors [488] there was a reduction in whole-body GPX activity in PP fed trout with Se-enriched yeast being more effective at raising these levels [489]. Se supplementation had no effect on hepatic catalase [490] glutathione reductase, glutathione-S-transferase or SOD activities [488]. Hepatic expression of *gpx1b2* and *gpx4a2* were up-regulated in fish fed Se-augmented diets [488] while in fry (~7.3 g), when fed the selenoyeast diet, the same group reported down-regulation of *CAT*, *Gclc* and *Nrf2* which correlated with activity levels of antioxidant enzymes [490].

Macrominerals (Ca, P, Mg, Na, K, Cl) play vital roles in fish physiological control processes and, when deficient, can cause a wide variety of problems ranging from cataract development, through to skeletal deformities, degeneration of pyloric enterocytes, anorexia, increased mortality, sluggishness, *inter alia*; [491] and [492] present comprehensive reviews of fish mineral nutrition. Alternative proteins present the possibility of dietary mineral deficiency, and this is illustrated by the work of [305] with trout who stressed the importance of ensuring an appropriate dietary balance. Even when mineral requirements are seemingly met these may change with age, environmental parameters and due to absorptive competition and binding within the gut lumen reducing bioavailability. Currently, there is a deficit of information regarding this important aspect of mineral nutrition which offers potential for future research.

3.11. Body composition

Although there are some exceptions, most trials undertaken with rainbow trout that compare FM, SCP and PP-based feeds report changes in whole-body and fillet/muscle composition. However, these effects are inconsistent and appear to vary not only with protein source, but with FI, age (perhaps reflecting more rapid growth rates of younger fish), selection pressure and trial length. Zhu *et al*. [296] found no difference in body composition when comparing trout fed a mixed PP *versus* FM-based feed as did [352] with CSM-based feeds and [377] evaluating a plant-based commercial feed. Yamamoto *et al*. [256] determined that supplementation of dSBM and CGM-based diets with EAA also resulted in fish having similar whole-body composition to animals fed FM, while [313] saw no effect of different vegetable oils on whole-body or fillet composition. When fed formulations containing distillers' grain or torula yeast (*Cyberlindnera jadinii*), whole-body composition also did not change [396, 493]. Nonetheless, fish in [493]'s trial expressed a higher ($p < 0.01$) viscerosomatic index (VSI) which supports the work of [257] who found that, irrespective of the presence or absence of supplemental Tau, PP diets heightened deposition of intraperitoneal fat but at a magnitude that failed to influence overall whole-body lipid levels. The effect of fish size on the dynamics of composition is illustrated with fSBM diets. Thus, fSBM resulted in lower fillet protein in 6 g fish [295], but had no discernable effects on fat, protein, moisture, or ash content in larger trout [299]. Another

example of age-related differences was seen with whole-body lipid levels which were higher in juveniles (10 g) fed PP diets but no different to control fish in larger (234 g) individuals [237].

SPC was reported to decrease whole-body lipid and dry matter content in 106 g animals ($P < 0.05$; [354]) while increasing protein content and decreasing lipid levels in 1.5 kg fish ($P < 0.05$; [357]). Trout fed a feed comprising 25% SPC among other PP ingredients, expressed whole-body protein and moisture levels that were higher ($P < 0.05$), and lipid and energy content lower ($P < 0.003$) than fish fed FM-based feeds [300]. FM-based feeds returned lower whole-body crude protein than SPC feeds but were alike PeP and RPC feeds [494]. Fillet fat levels were found to decline in trout fed a feed in which FM was replaced by pea protein [346]. Fry fed a bacterial SCP-based feed also expressed lower ($P < 0.05$) whole-body lipid and protein but higher moisture and ash than FM-fed animals [301]. Ash levels were likewise increased ($P < 0.01$) in trout fed protein-rich yeast at 10 and 15% [276]. PPC was found to increase only ash content by [277] but, in the studies of [236], PPC reduced whole-body dry matter, crude protein and fat composition while increasing ash in ~4 g. fish. A similar reduction in crude protein and fat levels ($P < 0.05$) and elevated muscle ash was reported in trout fed an enzymatically treated SBM [339]. In contrast, [230] observed that muscle protein was higher and lipid lower ($P < 0.05$) when comparing fish fed feeds containing blood meal *versus* those reared using PP diets. Muscle moisture and ash, however, were similar across feeds [230]. Both whole-body protein and lipid levels were reduced in fish fed a SCP-based feed [287] and lower fat, but higher protein and moisture content were observed in fish fed a fSBM feed [250]. A mixed PP-based diet, used in evaluating selection of fish for PP feeds had variable effects on whole-body lipid levels and protein digestibility [320] while whole-body fat content of rainbow trout fed mixed PP-based diets was significantly higher [495]. Replacement of FM with red lentil meal also increased lipid content while decreasing protein concentrations in both whole animal and fillet samples [366]. PP-based feeds resulted in higher ($P < 0.05$) whole-body protein content [267] and this was also witnessed for fillet protein and Ω-6 [284]. However, fillets had lower Ω-3 ($P < 0.05$) content. Dietary supplementation with *Nannochloropsis* sp., *Isochrysis* sp., and, or *Schizochytrium* spp. had no additive effects on DHA content of fillets – with only a feed containing all species of microalgae showing equivalence to a FM-based diet [496]. Increased body moisture ($P < 0.05$) was measured in fish fed algal-based feeds [283].

3.12. Quality

There are many ways to judge "quality" and different elements of the production chain assess quality traits using various and often distinct characteristics. Producers, processors, packagers and storehouses, wholesalers, retailers, marketeers and consumers each have their own notion of what summative attributes define quality. For processors, this may include fish size and shape, fillet and process yield, flesh color, fattiness and smoke/salt loss, and so on [497,498]. For consumers, quality encompasses all those attributes of a product that a purchaser anticipates. Because different people have distinct ideas of what characterizes product quality, definitions for this expression have become multifaceted but, in general, trout quality may be defined as the sum of those attributes that govern its acceptability to the consumer while conforming to the legal instruments of regulating agencies. Difficulties in judging trout quality are made more complex due to its wide variety of product forms. Trout are sold as pan-sized, fresh or chilled portions, headed and gutted (dressed), as fresh, chilled or frozen fillets and as processed product, including hot and cold smoked whole and filleted flesh and as

chopped and minced meat formed into sausages and burgers. Trout are also sold as jerky, in marinated, barbecued, roasted, and other preprocessed forms. Bottled, canned and dry pelleted trout are also available, including that for pet foods. Various techniques have been employed to assess fish quality including mechanical, chemical, instrumental, microbiological, molecular, and sensory methods [499–502] and some of these have been used to compare the impact of FM-, and PP-based diets on trout quality.

Failing to meet a consumer's expectations for a specific quality trait may result in a reduced willingness to pay for a product [23] and, in some studies, fillet color ranks second only to freshness as a critical quality attribute [503]. Fillet color is affected by diet-family interactions [504] and several studies have demonstrated that certain plant and SCP ingredients can influence fillet color. For example, [345] recorded differences in raw and cooked fillet color for trout fed SPC, with lightness (L) values being increased and redness (a) and yellowness (b) reduced. In contrast, [503] reported a decrease in L, with a (red) and b (yellow) increasing in trout fed a PP feed for 84 d when supplemented with 5 and 15% *Spirulina*. [267] determined differences in fillet L, a and b, with L and a being generally lower and b higher in PP-fed fish, resulting in flesh yellowing which was likely due to CGM used in the feed (even though astaxanthin was added). The authors, together with [230], conjectured that the yellowing resulted primarily because of CGM xanthophylls but also due to SBM components of the diet interfering with astaxanthin uptake and or deposition [267]. PP fed trout expressed a yellowed fillet in the studies of [370,371] and, although [286] measured fillet lightness (L) and yellowness being elevated and decreased respectively for PP fed fish, this was not perceived by a taste panel. Using the cyanobacterium *Arthrospira plantensis* to replace FM, [404] registered no changes in external coloration of fish, but a more intense yellow/orange hue for fillets which increased in yellowness upon cooking. The authors suggested that the yellowing was induced by *Arthrospira*'s carotenoid content. While this discoloration could be considered as a negative to consumers who preferred a white or pinkish-red fillet color in trout, the yellowing was also considered as "credence attribute" from a sustainability perspective [503] - fillet yellowing could be promoted as an indicator of sustainability in the marketplace.

Fish fed feeds in which FM was replaced by DGGY [493] over a 9-week period showed no effect on fillet yields; whereas, mixed PP-based diets have resulted in greater fillet yield [284]. A higher percentage dress-out has been observed in fish fed PP over a total production cycle [284], although over shorter periods (53 to 90-d) PP-based feeds had no such effect [285, 286]. Body composition and form can also be designated as components of breeding programs. For example, when comparing fish selected over ten generations, [408] found that trout fed on a FM-free feed expressed higher muscle fat, and headless carcass yield but lower VSI than comparably sized animals fed on a FM-based diet. Parisi *et al*. [371] examined the influence of production-length feeding on post-mortem changes in triploid (3n) rainbow trout (670 g, PP and 832 g FM). Fish fed PP feeds entered full rigor 2-h post-mortem, while trout reared on FM feeds entered full rigor 5-h post-mortem ($P < 0.05$). The change in cranial epaxial muscle adenylate energy charge – the ratio relating to ATP, ADP and AMP, was also lowered in PP fish 2-h post-mortem. Fillet length as a percentage of length measured at 0-h post-mortem, was lower ($P < 0.01$) for PP-fed fish from 2 to 4-h, after which there was parity. Parisi *et al*. [371] suggested that the observed biochemical changes measured in the PP fillet may have consequences for shelf-life and quality characteristics of the final product. Indeed, the latter suspicion appears to have been vindicated by the work of [504] who examined whether long-term feeding of trout on EPA/DHA-less PP-based feeds influenced spoilage during ice storage using

the quality index method and a trained taste panel. Quality scores were taken 3, 6, 9, 14 and 17-d post storage. The quality assessments determined that, when compared against fish raised on a FM-based and commercial-like feed, PP fed fish exhibited extra fresh characteristics until day 6 but, by day 9, ice-stored trout expressed poorer sensory freshness than the control groups and, by day 14, were deemed unacceptable.

Kaushik *et al*. [345] recorded increased shear strength in fish raised using SPC and like observations were made by [404] for trout fed *Spirulina*-based feed. In contrast, [286] failed to observe differences between trout fed PP or FM feeds, irrespective of the presence of guar binder. However, they saw no changes in cooking loss which contrasted to the observations of [404]. Sensory evaluation of fillets derived from fish grown using various PP-based formulations revealed differences especially in feeds that incorporated rapeseed oil [230]. These fillets exhibited the lowest scores for fishy flavor ($P < 0.05$) and higher tallies than most for chicken flavor. Interestingly, three of the PP-based feed fed trout returned a higher grade for fishy flavor than did the commercial FM-based feed ($P < 0.05$). The authors conjectured that differences in fish flavor may have resulted due to the use of different soy products employed. Kaushik *et al*. [345] reported that taste testing revealed that FM v. SPC fillets could be distinguished by their rancid and freshwater characteristics. Sensory analysis of trout fed PP feeds returned higher hardness, lower sweetness, and decreased odor intensity [367] while [337] found that fish were less ($P < 0.05$) tender and juicy than animals receiving FM-based feeds. Adelizi *et al*. [230] examined the impact of peanut meal, SBM, SPCs and SF on fillet flavor intensity and reported no differences in sweet, nutty, buttery or chicken flavors when compared against a commercial salmonid feed. However, there were some differences in flavor between test protein feeds: more intense fishy flavors were detected for SBM and SPC *versus* the control. Evaluation of 400 g trout fed for 88 days on CPC, SPC DDG *versus* those fed FM revealed that the latter had a firmer texture and fishier aroma and was preferred to the PP diets ($P < 0.05$; [393]). Noteworthy is that [229] reported that taste panel members gave higher preference to fish fed PP-based feeds for 56 d than trout raised on commercial feeds which the authors linked to reduced protein and fat and increased moisture levels. It should be recalled, however, that partialities of taste panels, and especially when comprising naïve testers, may be influenced by age, gender, culinary heritage, ethnicity and educational attainment. Clearly, quality profiles may be shaped using selection programs and with diets incorporating different ingredients and, using different blends of protein and lipid, may provide the means to produce designer fish, developed for specific markets and process needs [498,505,506].

3.13. Checking product authenticity

Consumers demand safer and higher quality fish and total transparency regarding the identity and characteristics of products. This is particularly true for seafoods which have become one of the most adulterated of all foodstuffs, leading to the development of many methods to trace and authenticate raw materials. Verification of fish provenance and handling history, including feed type used during cultivation, is important for labeling purposes and protects consumers from fraudulent practices. The ability to trace trout throughout a production chain represents a basic requirement for preventing illicit activities and protecting consumers, producers, processors, wholesalers and retailers alike. Techniques that allow verification of marine resource-free fed rainbow trout, therefore, become invaluable. Stable isotope analysis has been used for some time, especially to examine food

webs in ecological studies. The principle of the method relies on following the assimilation of the isotopes into animal tissues as they travel up a trophic chain. Commonly, $\delta^{13}C$ and $\delta^{15}N$ are used. Primary producers express distinct isotopic signatures and, as they are consumed, the signature merges into the consumer's tissues through fractionation which is caused by alterations in the heavy-to-light isotopic ratio. Stable carbon isotopes point to nutrient source while that of nitrogen, the consumer's trophic level—that is isotopic values reflect dietary composition and their differences in muscle can potentially be used to discriminate between marine resource- and plant-fed animals [507].

Cao *et al*. [508] examined the effect of replacing up to 50% of FM in trout feeds using mixtures of SBM and meat and bone meal (MBM) using nitrogen stable isotope analysis. Using this method, they were able to determine the impact and nutritional contributions of the three proteins by examining hepatic and dorsal muscle $\delta^{15}N$ values. As dietary FM content declined, so too did $\delta^{15}N$, while at lowest levels of incorporation, SBM (9%) and MBM (6.4%) provided 4.6 and 13% nutritional contributions, respectively. Similar responses were reported earlier [509] for FM replacement by poultry by-product in trout feeds. Moreno-Rojas *et al*. [510] examined stable isotope analysis as a method for distinguishing between trout fed principally PP and FM-based diets. Following a 103-d feeding trial no variations were seen in animal performance. However, examination of fillet $\delta^{13}C$ and $\delta^{15}N$ revealed significant ($P < 0.05$) differences for both isotopes with lower values for $\delta^{13}C$ and higher for $\delta^{15}N$ for the fish reared on the PP diet. The final values for $\delta^{13}C$ and $\delta^{15}N$ in both feeds reflected those of their ingredients. Comparable results were reported by [511] for trout fingerlings. As validation for the technique, it is noteworthy that studies with other species, in which diets employed mixed FM/PP ingredients or excluded all marine resources, provided acceptable dietary discriminations too [*e.g.*, 228,512–514].

Another method of checking authenticity of marine resource-free trout is through image analysis. Thus, Saberioon *et al.* [515] compared the effects of PP and FM-based feeds on trout skin color and texture following 21-days feeding. They used digital imaging of anesthetized fish and extracted 27 features–23 color and 4 texture–per image which were classified using four methods *viz.* Support Vector Machine (SVM), Random Forest (RF), Logistic Regression (LR), and *k*-Nearest Neighbors (*k*-NN). The authors evaluated the specificity and sensitivity of each model in identifying fish reared on the different diets and determined that SVM (100%) > LR (85%) > RF (70%) > *k*-NN (35%). Saberioon *et al.* [516–518] also examined the potential of hyperspectral imagery (HI) - the integration of spectroscopy and imaging, to discriminate between fish fed a FM and PP-based feed. Their study determined that full wavelength classification models exhibited better discriminatory power than digital photography when spectral pre-treatment used Savitzky-Golay and First Derivative algorithms to remove noise.

The major advantage of using isotope analyses to verify product authenticity relates to its broad use and acceptance as a method. The technique also allows for the incorporation of other isotopes as a means of verifying feeding practices. Over the last few decades there has also been significant advances in instrumentation with improvements in sensitivity, detection limits, precision and accuracy. The method has also witnessed increasing and successful application to a widening variety of fish species. A major drawback of the method, however, is its cost which has underpinned the search for alternative, less expensive methods. Imaging techniques represent a more amenable method for ascertaining authenticity of PP-fed fish. While SVM classification methods proved to be accurate as a means of discriminating between fish of different feeding histories using color, the method may not be suitable for species that do not express variation in skin coloration. Ultimately,

enhanced prouct characterization will be refined using multiple methods of authentication, the selection of which will be dependent on cost, ease of use, accuracy and accessibility, not only of equipment but also of personnel capable of employing such and analyzing results.

3.14. Hematology, health and immunity

A wide range of extrinsic and intrinsic factors can cause disturbance to the normal hematology of fish, and trout are no exception to this rule [519–522]. Hematological parameters are commonly used to indicate fish physiological and health status, and include hematocrit, hemoglobin concentration, white blood cell counts, differential leukocyte counts, and measurements of various biochemical parameters (e.g., glucose, protein, hormones, enzymes, ions) and, more rarely, thrombocyte counts and blood cell morphology [523–526]. PP-based feeds are reported to have variable effects on rainbow trout blood indices. For instance, hematocrit decreases ($P < 0.05$) in trout fed various blended PP-based feeds when compared to FM-based commercial feed [230,235,378,339] or remain unaltered in fish fed dSBM and CGM + EAA ($P < 0.05$; [256,313]). Dabrowski and colleagues [328,378,527] suggested that a reason underlying decreased hematocrit in fish fed on CSM may have been due to the adverse impact of gossypol on the intestinal uptake of iron and, or formation of hepatic gossypol-iron complexes. Haghbayan and Mehrgan [339] and [256] also reported reduced availability of iron, as evidence by lower hemoglobin levels, which may potentially have heightened erythrocyte fragility, leading to reduced RBC counts and increased circulating WBC. In their case, [339] considered the decreased hemoglobin levels to be a result of phytic acid binding of iron.

Diets consisting of dSBM and CGM + EAA had no effect on circulating calcium, glucose, or triglyceride and total protein levels, but did heighten circulating P ($P < 0.05$; [256]). In other trials with 100% PP-based feeds no impact on circulating cholesterol or triglycerides were measured [313,447]. However, others report lower triglyceride with yeast-supplemented and full PP feeds [250,396]. PP-based feeds have also been associated with lowered plasma cholesterol [237,293,302,346,386]. Glucose levels have been reported as being stable [293,388], decreased [302,386] or elevated ($P < 0.001$) [346]. Other impacts of PP feeds include no effect on lipase [313], glutamic oxaloacetic transaminase (GOT) or lactate dehydrogenase (LDH), but declining glutamic pyruvic transaminase (GPT) and alkaline phosphatase (ALP; $P < 0.01$) activity [346]. Hang *et al*. [250] reported that fSBM fed trout had lower ($P < 0.05$) levels of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Dietary supplementation with Met/Lys and/or bile acids elevated ALT to normal levels, but those of AST remained suppressed [250]. Inclusion of RSM in trout diets has been demonstrated to cause disruption of thyroid function resulting in perturbations in circulating concentrations of T_3 and T_4 [528, 529]. Plasma cortisol levels were observed to be higher in trout raised on a PP-based diet over a period of 84 days [388], suggesting some level of stress and potential for a negative effect on growth and the immune system. Indeed, Rumsey *et al.* [380] compared feeds formulated using SPC or SBM instead of FM and observed differential responses to serological and non-specific immune parameters of fish fed for 182-d. However, while there were changes ($P < 0.05$) in measured leukocyte counts, killing activity, phagocytic indices, and myeloperoxidase activity, fish differed significantly in weights (FM = 126 g., SPC = 46 g., SBM = 17 g.), which lessens the observations' worth. Conversely [276], in evaluating the inclusion of a protein-rich yeast fraction in PP feeds fed over 84 days, recorded no differences in the percentage presence of lymphocytes, thrombocytes, neutrophils or monocytes.

Lysozyme, also called muramidase or N-acetylmuramic hydrolase, is a bactericidal enzyme that hydrolyses the peptidoglycan moiety of bacterial cell walls. Lysozyme occurs in trout phagocytic cells, mucus, ova and serum. Its serum activity remains unchanged in fish fed mixed PP feeds [235] and PeP-based feeds [346]. But, when trout are fed a 50:50 *Spirulina*: soybean mix [398] or a 10–15% yeast fraction ($P < 0.009$; [276]) lysozyme activities decline, whereas inclusion of torula yeast into a mixed PP feed increases serum activities [396]. However, it is doubtful that the observed increase activity of lysozyme was a consequence of yeast inclusion because the enzyme has weak activity against mannoprotein and fibrous β-(1,3) glucans which are the main components of yeast cell walls. Kesbiç *et al*. [346] also measured a 2-fold increase in myeloperoxidase (MPO) when feeding mixed PPs and a similar MPO response was described by [380] for trout fingerlings fed a soy protein feed. MPO is a cationic leukocyte haloperoxidase, primarily active during host responses to pathogens and it has also been linked to modulating neutrophil activity during inflammation. Serum complement activity was unaffected by PP feeds [235]; whereas, (myelo)peroxidase declined substantively [276] which the authors contended was aligned to an observed decrease in gut inflammation. Corn gluten [353] and fSBM [269] had no effect on lymphocyte or macrophage activity, but [289] reported that solvent-extracted SBM at 60–89% of dietary protein, depressed head kidney macrophage activity and respiratory burst activity, suggesting suppression of the innate immune system. In direct contrast to the latter findings, addition of Tau (10 g kg^{-1}) to a PP diet enhanced head kidney macrophage respiratory burst activity in fingerling trout [399].

Fish fed PP-based feeds exhibited healthier fins [284] and this observation was suggested to indicate good condition although an association between it and the presence of dietary components, as suggested by [530], could not be confirmed. Perhaps the most decisive method of determining whether PPs impact the health and immunocompetence of trout is to compare survival following a challenge with pathogens. Jalili *et al.* [235] and [372] found no differences in survival when fish were challenged with *Yersinia ruckeri* or *Flavobacterium psychrophilum,* respectively. Indeed, the authors concluded that intestinal enrichment of bacteria may have increased resistance to infections.

3.15. Reproduction

The ability to effectively control reproduction represents the foundation stone of contemporary industrial aquaculture. Modern-day aquaculture, therefore, was not initiated until the 1930s, after Houssay [531] demonstrated induction of ovulation *via* hypophysectomy. Since that time hormonal control of reproduction has become more sophisticated and vital to the industry for production of out-of-season seed supply and genetic improvement programs [532]. Whether PP-based feeds impact reproductive success becomes a prominent issue because it appears that offspring from broodstock raised on PP respond favorably to PP diets [233]. Valuable insights have been gained from short- and long-term studies for hormonal profiles, egg and sperm viability and otherwise. For example, Dabrowski and colleagues [328,378,527,533,534], although incorporating 5% krill meal into PP-based feeds, reported on the effects of replacing FM with CSM in fish of ~250 g. The trials, which lasted between 5 months and 3 years, showed that the CSM diet had no effects on gonadosomatic index (GSI), sex steroids, fecundity, or fertilization rates in females, but did decrease egg weight ($P < 0.05$). In males, circulating levels of testosterone, 11-ketotestosterone, and 7,20β-dihydroxy-4-pregnen-3-one did not vary and seminal plasma protein, lactate dehydrogenase activity sperm concentrations, motility and fertilization rates remained stable.

Pereira *et al*. [233] observed that broodstock rainbow trout fed feeds containing solvent extracted or defatted SBM, produced spawn that expressed modified EAA profiles and, although retaining elevated levels of n-3 PUFAs, overall, FA profiles also changed, but without effect on fry survival. There was no impact of feed on circulating 17β-estradiol levels, but changes in the contour and concentrations of circulating vitellogenin ($P < 0.05$) occurred [233] as reported also by [345] using SPC-based diets. However, in the studies of [233], the number of females spawned was lower ($P < 0.05$) in the PP-based feed, although their relative fecundity was identical to that of FM fed fish. Lazzarotto [535] determined that trout reared throughout a production cycle on a mixed PP feed returned ova of lower weight at first and second spawnings ($P < 0.05$), with GSIs also being lower ($P < 0.05$). Absolute fecundity in terms of ova female^{-1} was greater in first spawning fish but survival to the eyed stage, through hatching and swim-up, were lower ($P < 0.05$). In second spawners the latter parameters were similar between the FM/FO-free and FM fed fish. There were no differences in total lipid content of ova or swim-up fry between feeds or years [234] but FA composition of alevins differed, showing higher percentages of 18:2n-3 and 18:3n-3 and a whole-body transcriptome that experienced significant effects on genes regulating lipid, carbohydrate and protein metabolism, muscle development and contraction, transport and catabolism after 3-weeks feeding [535,536].

More recent studies [537], that used monosex fish from first feeding, either on a mixed PP feed or commercial-like feed for 2 years, report that those fed on the commercial diet were heavier (+23%) and spawn egg weight greater (+33%). However, relative fecundity (egg g^{-1}) was 14-times greater in the plant fed fish, but GSI and egg weight and size were higher ($P < 0.05$) in the FM group. There were no differences in embryo development between diets, but fry weight was higher ($P < 0.01$) from FM fed broodstock. The maternal feed was found to influence DHA, EPA and ARA metabolism and surprisingly, given the lack of DHA, EPA and ARA in [537]'s plant diet, they were present in significant amounts in the eggs and fry of offspring, thereby illustrating preferential conservation and privileged biosynthesis. This is indicative of brood selectively concentrating PUFA into eggs, as found also by others [233,234,537]. This presumably ensures appropriate embryonic development during endogenous feeding. However, [323] cautioned that even given increased fecundity, high carbohydrate-reduced protein feeds of brood animals could still have negative consequences to offspring as evidenced by poorer survival. However, her earlier paper [322], recants this statement. Most of the findings on the reproductive performance of trout are likely due to the disruptive effects of phytoestrogens (Table 1). Estrogenic compounds, which are mostly isoflavones, have been reported for a wide variety of PPs, including soybeans and cottonseed, and can have diverse effects on various physiological processes as considered in [121]. However, there is little doubt that dose-related responses and differences in protein source and processing exist which may partly explain variations in observations.

3.16. Genetic factors

The natural geographic range of rainbow trout extends from the mouth of the Amur River, across to Alaska, the Yukon and British Columbia, and south to west central Mexico. This broad pattern of distribution, the species introduction to every continent except Antarctica [539–541], and widespread culture, ready hybridization and extensive release as a game fish and escapees, has resulted in significant ecological turmoil. This includes increased predation and competition with native fish, introduction of parasites and diseases into new areas and introgressive hybridization with

native salmonids, leading to loss of genetic integrity [542]. Nonetheless, the widespread distribution of rainbow trout has led to the existence of hundreds and more commercial and localized strains globally and several studies have determined that certain strains of rainbow trout respond better to PP-based diets than others. For example, [543] examined genotype x feed interactions in a commercial strain of trout and discovered large genetic variation for growth for both animals fed FM-based and wheat/corn gluten-based feeds. The authors suggested that their findings indicated that when strains expressed good growth on FM-based feeds they would likely also perform well on non-marine ingredient-based feeds (but see [329,349]). Barnes *et al*. [272] compared McConaughy and Shasta River strains fed on fermented soy products but found no difference between strains for growth, mortality or FCR, with both strains under-performing in terms of weight gain and FCR when compared against animals fed a FM-based feed. Introgression of eight strains of rainbow trout selected for faster growth on plant-based diets resulted in better growth and protein retention [364]. Callet *et al*. [538,544] compared three isogenic lines of trout that expressed similar growth when fed marine-based feeds but differed in their growth response to PP-based feeds from first feeding. Differences in strain growth were not due to FI but differences in FCE which was also associated with variations in hepatic transcriptome profiles and activation of pathways correlated with lipid and cholesterol metabolism. On the other hand, Brezas *et al*. [475] found no strain differences in nutrient digestibility between fish selected for PP feeds and a non-selected strain. In a selection program for enhanced ability to utilize PPs, [545] observed, after three generations of selection, that trout returned growth equivalence to those fed on a FM feed. This represented the elimination of a 36.8% deficit in weight gain before selection. Like results were recounted by [408] following ten generations of multi-trait selection. More recently, [406] examined the response of a PP selected line versus a non-selected strain to PP feeds and found differences in final weight, SGRs and FI favoring the selected line while [316] reported poorer ($P < 0.001$) survival, SGR and weight gain for non-selected fish when fed on PP feeds.

Gene expression analyses provide insights into normal and abnormal physiological processes and thereby provide a way to more completely understand basic molecular mechanisms involved in the response of organisms to their environment. In this respect, changes in patterns of gene expression in various tissues, following feeding with novel diets, has received increasing attention in rainbow trout. The impact of replacing FM/FO in trout feeds on gene expression has enhanced our understanding of gut and liver functions, muscle growth, immunity and metabolism. However, most physiological control processes are generally considered to be directed by several genes acting in concert, rather than one or a few and this is suggested by array technologies which permit the exploration of thousands of genes at one moment in time. As with the gut microbiome, gene expression profiles alone are of limited practical use although they do permit researchers to develop hypotheses regarding the nature of specific (groups of) genes. Confirmation of the importance of up- or down-regulated genes to animal physiology and performance, for example by linking profiles with histological and biochemical methods, will remain vital to applied nutritional research and enable a better understanding of the functional importance of different genes and how they may be regulated.

3.17. Immunity

As noted for protein digestion, expression of *PepT1* varies with dietary protein constituents and FI and PP-based diets induce higher levels of expression of immune system (IFN-γ) and

inflammatory (IL-8, IL-1β, TGF-β, TNF-α and TGF-β) genes in both the proximal and distal gut of trout. IL-8 and IL-1β are pro-inflammatory genes activated during the preliminary stages of an immune challenge. They stimulate proliferation of macrophages, T and B lymphocytes and the migration of neutrophils to sites of breaching and infection. The cytokines TNF-α and TGF-β are signaling proteins involved in chemotaxis whereas IFN-γ initiates macrophage activity, stimulating phagocytic activity. A mixture of these responses has been described in trout following feeding diets comprising SBM and WG [290] and a PP feed containing 36% *Chlorella sorokiniana* meal [282]. In the latter study, up-regulation of intestinal IgT was also observed. Taken together, these results undoubtedly indicate that some degree of gut inflammation was taking place. However, [346] reported that replacement of FM with PePI resulted in down regulation of hepatic TNF-α, but stable expression of IL-1β and IL-8 following 60 days feeding, suggesting that the gut's immune response to feed protein challenges (and probably feed intake) is variable. It is noteworthy that TNF-α has been implicated in the secretion of Cl^- into the intestine [546] which suggests that this cytokine may be involved in the progress of diarrhea often associated with PP-based feeds.

3.18. Gut function

Elevated IFN-γ expression accompanying reduced patency and function of the epithelial barrier would be expected to be associated with changes in tight junction-related genes and this has been observed for the tight junction proteins *tjp1a*, *tjp3*, *marveld1*, and *marveld3* in trout fed various PP-based feeds [447]. One might also anticipate increased mucus production under the circumstances of intestinal insult, and this is suggested for trout fed a protein-rich yeast-supplemented feed in which expression levels of gut mucin (*muc5ac*), together with genes associated with the junctional complex, occludin (*ocln*) and claudin (*cldn3a*) were up-regulated [276,396]. Increased expression of genes involved in cell proliferation and growth would also be expected and this has been reported for the hepatic insulin-nutrient-signaling pathway (*mTOR*, *rps6k1*, *AKT1* and *eif4ebp*) in trout maintained on a *Chlorella*-based feed [282]. In juvenile trout fed PP feeds from first feeding, Lazzarotto *et al*. [237] detected down-regulation of a variety of intestinal genes engaged in protein catabolism (e.g., *ctsl2*, *dpp7*, *folh1*), carbohydrate metabolism (e.g., *man2b1*, *fuca1&2*, *glb1, pfkfb3*) and trafficking, whereas hepatic genes implicated in lipid and cholesterol metabolism were up-regulated (e.g., *elovl2*, *cyp51a1i, dhcr7*, *tm7sf2*). However, because only a small number of genes were differentially expressed in the juvenile intestine and liver, diet-induced changes were considered as only slight. Other dietary manipulations, exemplified by increased carbohydrate levels [547,548] and replacement of FO with algal oil [549] in trout feeds have likewise been reported to impact gene expressions both positively and negatively, while [450] reported that higher temperature increased expression for genes involved in AA, fat, carbohydrate and energy metabolism and FA biosynthesis.

3.19. Hepatic metabolism

In contrast to observations for the intestine, [348] did not observe changes in the hepatic insulin-nutrient-signaling pathway (*Atk-TOR-S6K1*) following feeding of a PP feed, but the same group [369] observed PPs to differentially impact 75 hepatic genes, with 60 of these being up-regulated, representing four clusters of 16 to 26 genes each. These clusters were mainly involved in energy and protein metabolism. In an additional study [368] they determined that, when compared to a full

FM/FO-based feed, trout fed a complete PP diet returned differences in hepatic gene expression including up-regulation of those involved in metabolism (96 genes), cellular processes (14) and transport (10) and down-regulation in 74, 16 and 4 respectively. Nonetheless, these effects, like those observed by [237], were also considered as weak (max. +1.5-, and -1.3-fold changes), without any major impact on hepatic metabolism, cell cycling, stress, and welfare. Others, however, recognized more significant changes in the expression of hepatic genes involved in the same pathways [*e.g.*, 151,243,296,387,391,447–449,550] and [346] reported that PePI down-regulated expression of hepatic GH and IGF-I genes ($P > 0.05$). These contrasting findings suggest that different PP and SCP sources have variable metabolic effects that may be exaggerated further in the presence of other dietary ingredients and by factors such as fish strain, age, water temperature and quality. For example, saponins, present in SBM, LSM, PPC and others, are poorly absorbed and thus have extended gut residency times, interfere with digestive enzymes and form complexes with cholesterol and bile salts, increasing their evacuation from the gut [551]. The knock-on effects of this are reduced bile salt resorption and bile acid synthesis associated with the down-regulation of genes involved in bile acid synthesis (*cyp7a1, cyp7a1-2*, *cyp8b1-1* and *cyp8b1-2*) and cholesterol removal (*abcg8*) and increased expression of the bile acid synthesis inhibitor gene (*shp-2*) has been demonstrated in trout fed legume-based feeds [296,363]. These responses were accompanied by elevated expression of *srebp-2* which the latter authors took as evidence for increased cholesterol synthesis and reduction in cholesterol catabolism in efforts to restore cholesterol homeostasis. However, even given the significant neosynthesis of cholesterol, plasma and whole-body levels of plant fed fish didn't achieve those measured in the FM/FO fed trout [296] which, they conjectured, may be one reason underlying poor growth and reproductive performance.

3.20. Muscle function

Muscle growth and quality are critical because they influence profitability and consumer acceptance of the product. High quality muscle ensures superior texture, taste and nutritional value and, in fish, muscle growth occurs by hyperplasia and hypertrophy [552] which are regulated by a complex network of genes engaged in myogenesis, cell proliferation and protein metabolism [308,553–555]. These include myogenic regulating factors, such as myostatin (*mstn*) which inhibits proliferation of trout myoblasts [556], myogenic differentiation 1 (*MyoD1*), myogenic factor 5 (*MYF5*) and 6 (*Mrf4*) which are engaged in trout muscle development and differentiation [553], and the paired box (*Pax*) family [557] which play crucial roles in controlling the activation of muscle stem cells, and in the development and differentiation of myoblasts. When compared to terrestrial meat producers like chickens, swine and cattle, however, our understanding of the molecular basis of trout muscle growth and regulation is highly restricted. Linked to decreased somatic growth of fish fed PP feeds for 84-d, [308] determined a significant shift in the distribution of white muscle fiber diameters with trout exhibiting decreased median diameters when compared against fish fed FM-based feeds ($P < 0.0001$) and this was accompanied by an increase ($P < 0.01$) in white, but not red muscle cathepsin D (*ctsd*) expression, implying muscle-type *ctsd* sensing to changes in dietary protein source. This may be relevant to the preferential preservation of red muscle used for low-speed swimming. In [308]'s research, they did not see an effect of PP on the proteolysis marker calpain-2 (*capn2*), or proliferating cell nuclear antigen (*pcna*), myogenin (*myog*), or slow myosin heavy chain (*mhc*) expression in either red or white muscle, thereby indicating that changes in dietary protein and

muscle phenotype do not correlate with changes in gene expression for markers of activation, proliferation and fusion of satellite cells. Also, *myog* expression levels were similarly unaffected by a mixture of different PP in the study of [268]. Snyder *et al.* [248] observed a down regulation of cathepsin L (*ctsl*), calpastatin L (*cast*), muscle RING-finger protein 1 (*MuRF-1*) and proteasome 20s delta subunit (*psmd*) when feeding SPI-based feeds with or without supplemental AAs but an up-regulation of the motor protein *myod2,* the transcription factors forkhead box 01 (*foxo-1*) and *Pax-7* which are engaged in myogenic growth and differentiation when the SPI was supplemented with 0.7% Met, 0.31% Lys, 0.51% Thr and 1.05% Gly. The authors suggested that their results indicated a propensity for reduced muscle cell growth when compared to trout fed FM-based feeds - higher degradation rates of muscle protein. Identification of primary regulators and genes involved in trout muscle growth, fillet yields, and quality would be of extreme value to marker-assisted selection programs. Such possibilities have been suggested by [558,559] for PP tolerant trout (63 candidate genes) and [560] for welfare indicators of feeding stress (*saa*, *mpo*, *nos2* and *usp2*), while the recognition of [561] that decreased *myhc* and changes in glycolytic enzymes were related to trout flesh firmness suggests these as possible markers of flesh quality. However, for this to be realistic for muscle quality-based selection programs, greater investment will be needed to increase our current understanding of the molecular basis of trout muscle growth.

3.21. Nutritional programming

Geurden *et al*. [334] examined the impact of raising trout fry, from first exogenous feeding, for 21-days on PP or FM-based feeds to verify whether this strategy might have benefit to the acceptance and or utilization of PPs later during production. Fry were subsequently presented with FM-based feed for 196-d after which they were challenged with the PP feed for a 25-day period. Fish reared on the PP feed during first exogenous feeding expressed ~42% higher SGR when compared with fish initially reared using FM-based feed. The additional weight gain correlated with higher FI ($P > 0.0001$) and FE ($P < 0.003$), combined with increased protein and lipid gains, thereby suggesting early exposure to a specific dietary formulation could have lasting physiological effects later in life. Cardona *et al*. [324] examined this potential further by feeding female broodstock a plant/algal (*Schizochytrium* sp.) diet and evaluating the performance of their offspring. They reported improved growth of 4-month-old progeny challenged with a similar diet with an increased capacity for endogenous biosynthesis of cholesterol and LC-PUFAs associated with increased expression of genes involved in cholesterol synthesis. The authors suggested that the enhanced ability of offspring to biosynthesize cholesterol likely resulted as an expectation of dietary cholesterol deficiency. Early, temporary feeding of PP-based feeds to first exogenous feeding trout fry allows them to assimilate a flavor and feed partiality that is operational as juveniles. This preference is accompanied by increased FI, growth and feed efficiency and, in efforts to explain the mechanisms underlying this nutritional programming, Balasubramanian *et al.* [309] used gene expression analysis to identify genes and pathways engaged in food predilection in the brain and liver. In the brain, they isolated transcriptomic changes in pathways dealing with sensory perception, Met metabolism, and neuroendocrine peptides in regulating feeding responses. In the liver, they revealed that early exposure to PP feeds impacted genes involved with the mediation of intermediary metabolism, proteolysis, and protein folding.

4. Summary and conclusions

Forage fish catches have been stagnant or on the decline since the 1980s [100,562,563]. Moreover, the use of forage fish in aquafeeds has become heavily criticized due to its negative impact on marine ecosystems and the deflection of these species away from human consumption [101,564]. This has led to research focused on reducing the reliance of fed aquaculture on marine resources with particular attention to carnivorous species such as salmonids [50]. Given the citations herein, there can be no doubt that rainbow trout, from hatching through to harvesting, irrespective of final size, can be raised on feeds totally void of FM when replaced by vegetable and SCPs. In essence the studies presented punctuate the statement of [53], and many researchers since, that trout do not require specific dietary ingredients but feeds that satisfy the species' nutrient needs and energy requirements. Nonetheless, performance penalties do accrue when single ingredients supplant dietary FM. However, improved growth and digestibility can be attained with prudent amalgamations of feed ingredients, perhaps resulting due to a more balanced nutritional profile, reductions in ANFs and their secondary metabolites, nutritional signaling and otherwise. Future research is still needed to determine ingredient complimentarity to improve utilization and optimize fish performance. Better production has also been attained when PPs receive pre- or additional processing, increasing crude protein content and removing ANFs [3,565]. Nevertheless, extra processing steps increase costs which lead to diets that are more expensive than commercial FM-based feeds. Alternative PP ingredients may become more competitive, as seems to be the case with certain SCPs with scaling, following selective breeding of candidate ingredients to reduce or remove ANFs [3,566]. These approaches need to be combined with innovations in ingredient and feed processing technologies including taking account of how novel formulations may influence, for example, AA nutrition. While research has started to consider these issues, more studies are needed, especially on defining what specifically corresponds to essentiality and how so-called nonessential or conditionally essential nutrients contribute to gene expression, growth and other physiological control processes [567–569]. Similar research is needed to ensure optimal mineral nutrition with amalgamated alternative feedstuffs. Novel processing techniques have allowed the incorporation of a broader variety of ingredients while reducing or eliminating ANFs, enhancing feed palatability and nutrient digestibility. Any proposed alternative protein must undergo robust vetting across the board and it is imperative that PP and SCP-based feeds do not negatively influence either animal well-being nor product quality, shelf-life or consumer acceptability. Present evidence indicates that there is room for intensified research and improvements in all the above arenas. Clear is that a multidisciplinary research approach will be required to more rapidly achieve the goal of acceptably eliminating marine resources from trout feeds.

Acknowledgement

The authors are indebted to the Anthropocene Institute, Palo Alto, CA94301, USA, for their sustained, ardent support.

Use of AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

Conflict of interest

The authors declare that they have no conflicts of interest. Ewen McLean is an editorial board member for *AIMS Animal Science* and was not involved in the editorial review or the decision to publish this article.

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