Norwegian University of Life Sciences
Faculty of Biosciences
Department of Animal and Aquacultural Sciences

Philosophiae Doctor (PhD)
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Abbreviations and definitions

BSF -Black soldier fly larvae
BSFC+ -A non-processed black soldier fly larvae meal produced for *Paper II*.
BSFC- -A dechitinated black solider fly larvae meal produced for *Paper II*.
BSFC+P+ -A black soldier fly larvae meal fermented with *Pediococcus acidilactici* for *Paper II*.
DHA -Docosahexaenoic acid, a polyunsaturated omega-3 fatty acid.
EPA -Eicosapentaenoic acid, a polyunsaturated omega-3 fatty acid.
ES -Ecosystem services
ETS -Enzyme treated soybean meal used in Paper I.
FM -Fish meal
FW -Fresh water
FOS -Fructooligosaccharide
GI -Gastro-intestinal
H&E -Haematoxylin and eosin stain using in histological methods.
LAB -Lactic acid bacteria
LCA -Life cycle analysis
MOS -Mannan oligosaccharides
MIX -A mix of commercially used aquafeed plant proteins formulated for *Paper III*.
NSP -Non-starch polysaccharides
PCR -Polymerase chain reaction
PUFA – Polyunsaturated fatty acids
SBM -Soybean meal
SBMIE -Soybean meal induced enteritis
SDG -Sustainable development goals
SPC -Soy protein concentrate
SPI -Soy protein isolate
SW -Saltwater

Summary

Aquaculture has emerged as the fastest growing food production system globally and will play an important role in future food security for a growing population. Atlantic salmon (*Salmo salar*) is an intensively farmed and highly domesticated finfish and as a carnivore presents a challenge to the feed sector. Traditional formulated feeds were reliant on fish meal (FM) from marine sources, but long-term over exploitation of wild fisheries, and rising ingredient costs necessitated transition. Soybean meal (SBM), a cheap, scalable, and high protein product, was used alongside other plant proteins, to partially replace FM. However, increasing demand for SBM, and detection of adverse impacts to salmon gut health led to further processing of soybean to produce ingredients such as soy protein concentrate (SPC), which while widely used in modern aquafeeds is still limited. To address this bottleneck, aquafeed research seeks to identify nutritionally compatible and scalable alternative proteins. Insect and microbial ingredients have emerged as promising candidates for Atlantic salmon feeds. They can be produced independent of location, and with low carbon footprint on a wide variety of substrates. Yet, there are knowledge gaps for characterising the impact and optimising application for health and welfare of farmed salmon. Furthermore, to ensure the sustainable exploitation alternative and protein sources, supply chains must be resilient supply to future-proof the aquaculture sector. Three research priorities were identified from knowledge gaps in the existing literature. Firstly, that there was a need to characterise and optimise the proteins that are currently available to the aquafeed sector, secondly, that there was a need to characterise and optimise alternative proteins that have potential for the aquafeed sector, and thirdly, that there is a need for environmentally sustainable mechanisms to exploit new protein sources. The aim of this thesis was to address these priorities, and this was done through three multi-discipline research studies evaluating growth performance, gut microbiome and behaviour in juvenile Atlantic salmon and a review study.

Paper I addressed the first priority and investigated gut-targeted enhancements of SBM, enzyme pre-treatment and addition of fructooligosaccharide. The impact to growth performance, gut microbiome, and behaviour compared to conventional

SBM and FM was evaluated. SBM was included at 25% in all SBM diets. Enhancement provided benefits to growth performance and altered the gut microbiome community, increasing the presence of *Enterococcus* in fish fed enzyme pre-treated SBM. This study also indicated for the first time the differing impact of marine protein and plant protein on the behaviour measure of coping style, revealing that FM fed fish tended to be more proactive and may experience better welfare in intensive farm conditions. *Paper II* and *Paper III* addressed the second priority. *Paper II* investigated two novel processing methods of black soldier fly (*Hermetia illucens*) larvae (BSF), dechitination and fermentation with *Pediococcus acidilactici*. BSF was included at 10% in all BSF diets. The impact on growth performance, gut health, and gut microbiome compared to convention proteins was evaluated. All diets supported comparable growth performance and gut health, but the gut microbiome was significantly altered by protein source and BSF processing method. The microbiome of fish fed fermented BSF were exclusively dominated by the chitinase-active bacterium, *Exiguobacterium*, and the chitin present in unprocessed BSF had a prebiotic potential, indicating chitin is beneficial at this inclusion level of BSF. *Paper III* investigated increasing inclusion levels, (0%, 10% and 20%) of torula yeast (*Cyberlindnera jadinii*) as a replacement for FM in a marine protein diet, and as a replacement for plant proteins in a mixed protein diet, and the impact on growth performance and gut microbiome was evaluated. When torula yeast replaced FM, comparable growth and gut microbiome communities were found, with an increase in favourable bacteria in the gut with increasing inclusion. However, when torula yeast replaced plant proteins, it was observed that 10% inclusion was optimal for growth performance, and that at 20% inclusion, potentially adverse changes were detected in the gut microbiome, such as a decrease in lactic acid bacteria. This study highlighted the importance of both optimising inclusion and combination of proteins in formulated diets. *Paper IV* addressed the third priority and mapped the ecosystem services (ES) provided by seaweed, perennial grasses, and legumes, under-utilised biomasses in Europe with aquafeed potential, and evaluated their potential for utilisation in aquafeeds. All these biomasses provide valuable ES and could be sustainably exploited by the aquafeed sector through circular bioeconomy mechanisms. Collectively these studies highlighted the importance of multi-disciplinary approaches to improving the aquafeed sector. From these key findings, new priorities from three focus areas for future research were outlined to support the long-term sustainability of Atlantic salmon aquaculture.

Norsk sammendrag

Akvakultur er den mest hurtigvoksende matproduserende næringen globalt, og den kommer til å spille en viktig rolle for å sikre matforsyningen til en stadig økende befolkning. Atlantisk laks (Salmo salar) utgjør en stor del av produksjonen i havbruksnæringen, men som opprinnelig karnivor fører den til flere utfordringer for fôrindustrien. Tradisjonelle fôrblandiner var i stor grad basert på fiskemel, men langvarig overforbruk av villfisk, samt økende råvarekostnader førte til at det var nødvendig med overgang til alternative proteinkilder. Soyamel (SBM), som er billig, tilgjengelig i stor kvanta og har er et høyt proteininnhold, ble benyttet sammen med andre planteproteiner for å delvis erstatte fiskemel, men kombinasjonen av en økende etterspørsel etter SBM kombinert med negative effekter på laksens tarmhelse førte til at soyabønnene ble videre prosessert til soyaproteinkonsentrat. Tilgjengeligheten av soyaproteinkonsentrat er noe begrenset, og for å unngå denne flaskehalsen forsøker mye av dagens forskning å finne alternative proteiner med tilsvarende næringsinnhold og muligheter for oppskalering. For tiden er proteiner fra insekter og mikroorganismer lovende kandidater som kan produseres med lavt karbonfotavtrykk, men kunnskap om hvordan disse påvirker velferden til oppdrettslaks er fortsatt mangelfull. I tillegg må forsyningskjedene stabiliseres for å sikre bærekraftig bruk av disse proteinene.

Basert på tilgjengelig kunnskap ble tre prioriterte forskningsområder identifisert: 1) å beskrive og optimalisere eksisterende proteinkilder, 2) å beskrive og optimalisere alternative proteinkilder, og 3) å utvikle mekanismer for å sikre bærekraftig bruk av nye proteinkilder. Denne artikkelen har som mål å styrke kunnskapen på disse fokusområdene gjennom tre tverrfagligeforsøk (I, II & III) med fokus på tilvekst, tarm-mikrobiologi og atferd hos atlantisk lakseyngel, samt en oversiktsartikkel (IV).

Artikkel I undersøkte det første fokusområdet ved å sammenligne bruken av standard SBM med bruk av henholdsvis enzymbehandlet SBM og SBM tilsatt FOS (fruktooligosakkarider). Alle SBM-diettene inneholdt 25% SBM, og effekten på vekst, tarm-mikrobiom og atferd ble undersøkt. Disse behandlingene resulterte i økt tilvekst, og et forandret tarm-mikrobiom, vist ved en økning av Enterococcus i den enzymbehandlede gruppen. I tillegg viste studien for første gang en forskjell mellom

proteinråvarenes effekt på atferdsmålet «coping style» (mestringsstil), ved at laks som fikk fiskemel hadde en mer proaktiv atferd, noe som kan føre til bedre fiskevelferd i oppdrettssituasjoner.

Artikkel II og III tok for seg det andre fokusområdet ved å undersøke forskjellige produksjonsprosesser for svart soldatflue (Hermetia illucens) (BSF) mel, d.v.s. fjerning av kitin, og fermentering med Pediococcus acidilactici. Alle BSF-dietter inneholdt 10% BSF. Effekten av disse behandlingene på tilvekst, tarm-mikrobiom og atferd ble sammenlignet med konvensjonelle proteinkilder. Det var ingen signifikante forskjeller i tilvekst eller tarmhelse, men tarm-mikrobiomet ble signifikant påvirket av både type proteinkilde og de ulike behandlingsmetodene. Tarm-mikrobiomet til laks som ble fôret med fermentert BSF ble dominert av Exiguobacterium, mens uprosessert BSF hadde et økt prebiotisk potensial som følge av økt kitininnhold.

Artikkel III undersøkte økende mengde (0%, 10% og 20%) av Torulagjær (Cyberlindnera jadinii) som erstatning for FM i en diett basert på marint protein, og som erstatning for planteproteiner i en diett basert på en blanding av planteproteinråvarer. Effekten på tilvekst og tarm-mikrobiom ble undersøkt, og resultatene var sammenlignbare når Torulagjær erstattet FM, og i tillegg ble det observert økt andel av gunstige tarmbakterier med økende mengde gjær. Derimot, når Torulagjæren erstattet planteproteiner, var 10% innblanding optimalt for vekst, mens 20% førte til mulige negative endringer i tarm-mikrobiomet, deriblant en reduksjon i melkesyrebakterier. Denne studien viser viktigheten av optimalisering av både inklusjonsnivå og proteinsammensetning i fôr.

Artikkel IV undersøkte det tredje fokusområdet og kartla økosystemtjenester (ES) for tare, flerårig gress og leguminoser; underutnyttede biomasser med potensiale for bruk i fôr. Studien viser at disse biomassene ga gode ES og kan benyttes på en bærekraftig måte i fôrindustrien gjennom sirkulær bioøkonomi.

Basert på resultatene fra ovennevnte artikler skisseres nye fokusområder og prioriteter for å legge til rette for bærekraftig oppdrett av laks.

List of papers

This thesis is based on the papers listed below. The papers will be referred to by their roman numerals throughout the thesis.

I- **Leeper, A.**, Sauphar, C., Berlizot, B., Koppe, W., Knobloch, S., Skírnisdóttir, S., Björnsdóttir, R., Øverland, M., and Benhaïm, D. Enhancement of soybean meal alters gut microbiome and influences behaviour of farmed Atlantic salmon (*Salmo salar*). (Manuscript).

II- **Leeper**, **A.**, Benhaïm, D., Smárason, Ö, B., Knobloch, S., Ómarsson, L, K., Bonnafoux, T., Pipan, M., Koppe, W., Björnsdóttir, R and Øverland, M. Feeding black soldier fly larvae (Hermetia illucens) reared on food waste alters gut characteristics of Atlantic salmon (*Salmo salar*). Journal of Insects in Food and Feed (Accepted September 2021).

III- **Leeper**, **A.**, Ekmay, R., Knobloch, S., Skírnisdóttir, S., Varunjikar, M., Dubois, M., Smárason, Ö, B., Árnason, J., Koppe, W and Benhaïm, D. Torula yeast in the diet of Atlantic salmon (*Salmo salar*) and the impact on growth performance and gut microbiome. Scientific reports (Accepted October 2021).

IV- Nielsen, K, C*., **Leeper, A***., Juul, L* and Øverland, M. The potential of green and blue protein systems for support a sustainable, circular bioeconomy in the aquafeed industry. Frontiers in Sustainable Food Systems (Submitted August 2021). * Denotes equal contribution.

1 Introduction

1.1 General Introduction

Global human population is expanding rapidly, thus, there is a need to ensure food security for a predicted 9 billion people by 2050 (Béné et al., 2015; Godfray et al., 2010). Food systems will need to be resilient and future-proofed to adapt to climate change while simultaneously limiting impact (Rockström et al., 2020). Aquaculture is the fastest growing food production sector in the world (Anderson et al., 2017) and it has a lower carbon footprint (Nijdam et al., 2012), and a greater potential to expand than terrestrial animals or capture fisheries to meet the rising protein demand for human consumption (Béné et al., 2015; Godfray et al., 2010). Commercial Atlantic salmon (*Salmo salar*) production has rapidly evolved from a few thousand tonnes in the 1980s to more than 2.4 million tonnes globally in 2018, led globally by Norway and Chile (Asche et al., 2013b; FAO, 2020). This growth has been driven primarily by advancements in nutrition, health, genetics, technological innovations, and high market demand (Asche et al., 2013b; Brækkan and Thyholdt, 2015; Torrissen et al., 2011).

The growth in this sector presents several environmental challenges. For the carnivore Atlantic salmon, foremost of these challenges is sourcing sustainable aquafeed ingredients to meet their high dietary protein demands (Naylor et al., 2009; NRC, 2011). Traditionally, the salmonid aquafeed industry has relied on raw materials from wild marine resources to produce fish meal (FM)(Naylor et al., 2009; Tacon and Metian, 2015). These resources are highly palatable, historically low-cost and they are compatible with the nutritional requirements of Atlantic salmon, and thus, were considered a “gold standard” (Turchini et al., 2019). In 1990, marine protein contributed to more than 65% of salmon feed composition (Shepherd et al., 2017). Long term over-exploitation of wild capture fisheries has led to increasing cost of these raw materials combined with increasing pressure to improve environmental sustainability (Jannathulla et al., 2019; Naylor et al., 2021), which led the industry to adopt low FM feeds (Olsen and Hasan, 2012). As an alternative, plant-protein sources such as soybean meal (SBM) and other plant ingredients such as rapeseed meal, pea protein concentrate and sunflower meal that were scalable to the growing industry were applied as replacements for FM (Gaitlin III et al., 2007). These feed ingredients, while initially highly available and cheaper than FM, have been shown to contain anti-nutritional factors (ANFs) which when fed in certain combinations can cause inflammation in the distal intestine of Atlantic salmon known as enteritis (Dale et al., 2009; Krogdahl et al., 2003). In modern Atlantic salmon diets despite the low inclusion of 30% marine protein sources (Ytrestøyl et al., 2015), pressure on wild fisheries remains high (Shannon and Waller, 2021). Though SBM has largely been replaced with soy protein concentrate (SPC), sunflower meal, faba beans and pea protein concentrate (Shepherd et al., 2017). Use of such plant ingredients is suboptimal for salmon, leads to intensified crop production and increased pressure on the environment and competes with the human consumer market creating bottlenecks and challenges for feed ingredients (Asche et al., 2013a; Goldsmith, 2008; Green et al., 2013; Heron et al., 2018; Prudêncio da Silva et al., 2010). Despite this, soy derived products remain the current most viable option for the aquaculture sector, therefore, the first priority of this thesis is to improve their application.

The second priority however, is to identify, characterise and improve the application of alternative proteins sources (Hua et al., 2019). Promising candidates include insect proteins (Barroso et al., 2014; Henry et al., 2015; Weththasinghe et al., 2021b), microbial ingredients such as yeasts (Glencross et al., 2020; Jones et al., 2020; Øverland and Skrede, 2017; Tlusty et al., 2017), and bacteria (Øverland et al., 2010), microalgae (Shah et al., 2017), marine macroalgae (Øverland et al., 2019) and terrestrial crops (Gaitlin III et al., 2007). These ingredients offer an opportunity to address the economic vulnerability induced by a European protein deficit (Smárason et al., 2019) by supporting more localised production (Tacon et al., 2011) and climate-resilient options for the aquaculture sector. While many of these ingredients have been evaluated in diets for Atlantic salmon, the impact of using a combination of these protein sources on key aspects of health and welfare is lacking. Furthermore, for many novel ingredients, a bottleneck exists due to the limited scale of production (Hua et al., 2019), high costs and incomplete realised supply chains (Mitra, 2020; Regueiro et al., 2021), highlighting the third priority of this thesis.

1.2 Background and status of knowledge

1.2.1 Soy derived products in salmon aquaculture

Soybean is one of the world´s largest agro-commodities, up to 335million tonnes was produced in 2016 and is produced extensively in global locations such as Latin America and The United States (Heron et al., 2018). Soybean is considered a high-quality protein source with a favourable amino acid profile and it is widely used in animal feeds, and increasingly in direct human consumption (El-Shemy, 2011; Lusas and Rhee, 1995; Messina, 2010). SBM contains about 48% crude protein, is compatible with aquafeed extrusion (Hardy, 2010) and became the most important plant protein source in modern aquafeeds (Kumar et al., 2020; Naylor et al., 2009; Tacon et al., 2011). Yet nutritional and health related bottlenecks associated with SBM limit its application in feeds particularly for salmonids.

When only minimal processing is applied, inclusions as low as 10% SBM (Krogdahl et al., 2003) can trigger soybean meal induced enteritis (SBMIE) in the distal intestine of Atlantic salmon (Baeverfjord and Krogdahl, 1996), and Chinook salmon (*Oncorhynchus tshwyscha*) (Booman et al., 2018) and to a lesser extent in Rainbow trout (*Onchorhynchus mykiss*) (Refstie et al., 2000). SBMIE alters the morphology of the distal gut by increasing the thickness of the submucosa, the connective tissue, the inflammatory cell infiltration into the lamina propria, and shortened mucosal folds and microvilli (Baeverfjord and Krogdahl, 1996; Krogdahl et al., 2000). Such changes displayed in **Figure 1**, can be detected after only 7 days of exposure (Romarheim et al., 2011), and can worsen with increased inclusion levels and higher temperatures (Krogdahl et al., 2003; Urán et al., 2009, 2008). The symptoms of SBMIE appear more severe in post-smolt Atlantic salmon gut than in the juvenile gut, potentially due to an under-developed immune system at early developmental stages which is incapable of a full inflammatory response. In comparison, in seawater the gut is more developed and more permeable, which increases the exposure to luminal antigens (Sahlmann et al., 2015). The consequences of SBMIE for the fish are a reduced nutrient digestibility and absorption, reduced intestinal immunity (Booman et al., 2018) and increased epithelial permeability of the gut wall, reducing its protective barrier function (Knudsen et al., 2008). SBM in aquafeeds also causes adverse changes to the gut microbiome community assemblage (Kononova et al., 2019), which has important indirect consequences for immune development, and fish resilience (Talwar et al., 2018).

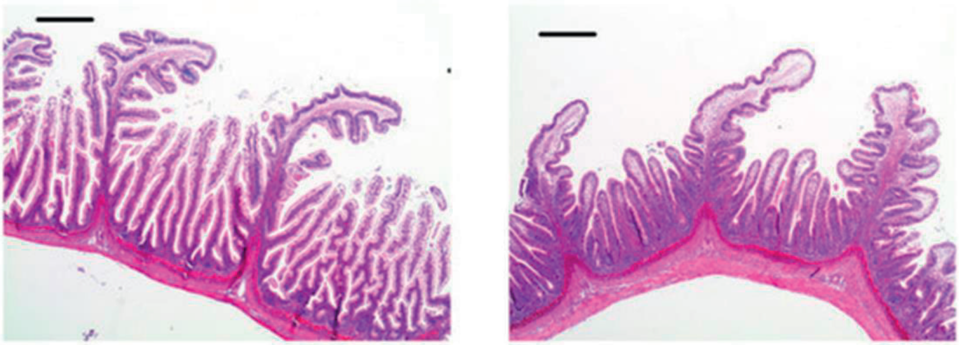

Figure 1. Microscopy of the distal intestine Atlantic salmon, histological samples were stained with hematoxylin and eosin. Image left is from a fish fed FM diet and image right is from a fish fed SBM (200g kg^{-1}) diet from (Romarheim et al., 2011). The fish fed SBM displayed features of SBMIE.

The driver of this inflammation is thought be anti-nutritional factors (ANF) present in soy protein, in particular soy saponins (Hedrera et al., 2013; Knudsen et al., 2008) and non-starch polysaccharides (NSP) (Ao and Choct, 2013). Similar ANF impacts have been detected in Atlantic salmon when they are fed other minimally processed plant proteins (Krogdahl et al., 2010). Saponins are heat-stable compounds that have a carbohydrate moiety joined with a triterpenoid or steroids (Krogdahl et al., 2015; Shi et al., 2004), and reduce the digestion of protein and fat, and increase the permeability of the intestinal wall (Gu et al., 2014; Knudsen et al., 2008). This has been associated with deleterious effects such as reduced barrier function (Hu et al., 2016). NSPs are remnant dietary fibres and are not well digested by salmonid endogenous enzymes or their associated gut bacteria, limiting nutrient uptake and reducing feed efficiency (Dalsgaard et al., 2016; Gaitlin III et al., 2007; Krogdahl et al., 2005). In response to the adverse impact to these characteristics of SBM, the aquafeed industry has switched to more highly processed ingredients derived from soybean, primarily soy protein concentrate (SPC) where soybean meal is refined by alcohol-washing (Green et al., 2013). SPC has a crude protein level of ~75%, which is much higher than SBM (Hardy, 2010). Alcohol-extracted SPC does not produce an inflammatory response in the distal gut of juvenile Atlantic salmon even when it replaces 80% of FM (Metochis et al., 2016), however at higher seawater temperatures, SPC has also been associated with intestinal disorders for salmonids (Desai et al., 2012; Green et al., 2013).

1.2.2 Alternative proteins from insects and yeast

Alternative proteins from insects and yeast have been identified as key promising candidates, and have received increasing attention as partial replacements for conventional proteins (Agboola et al., 2020; Barroso et al., 2014; Hua et al., 2019; Øverland et al., 2013). Both have nutritional profiles suitable for inclusion in feeds for Atlantic salmon and provide potential solutions for sustainable and resilient sources of protein for the aquaculture sector (Sánchez-Muros et al., 2014).

Insect proteins have a long history in aquafeed research (Makkar, 2016). Until recently, however, the low cost of conventional ingredients and European legislation limited the scale of production and use of insects as a protein source for commercial Atlantic salmon feeds (Askarian et al., 2012; English et al., 2021; Fisher et al., 2020; Tomberlin and van Huis, 2020; Weththasinghe et al., 2021b). In 2017, the European Union approved insect meals reared on authorized substrates for inclusion in aquafeeds (EU regulation 2017/893) (Hua et al., 2019), consumer acceptance is on the rise (Verbeke et al., 2015), and a number of larger scale commercial insect producers have become established, re-igniting the interest from the aquafeed sector. Viable insects include, house fly maggot (*Musca domestica*), mealworm (*Tenebrio molitor*) and black soldier fly larvae (*Hermetia illucens*) (BSF) the latter of which has been widely researched for suitability for salmonid diets (Belghit et al., 2018; Fisher et al., 2020; Henry et al., 2015; Makkar et al., 2014; Weththasinghe et al., 2021b). BSF has an average of 40-44% (in dry matter) crude protein content (Makkar et al., 2014).

Existing studies evaluating BSF in Atlantic salmon diets have indicated different optimal inclusion levels, with some studies suggesting that 100% of FM can be replaced with BSF in the post-smolt stage (Belghit et al., 2019). Other studies recommend lower inclusion levels between 5-25% (Henry et al., 2015) and 12.5% (Weththasinghe et al., 2021a). Contradictory results in the literature exist regarding possible adverse impacts of BSF on fillet sensory characteristics in Atlantic salmon. Some research reports no impact (Bruni et al., 2020; Lock et al., 2016) while others report an undesirable rancid flavour when BSF replace 100% of FM (Belghit et al., 2019). This will be important to elucidate to facilitate consumer acceptance and market uptake (Calanche et al., 2020). Evidence indicates that BSF has favourable digestibility for salmon (Fisher et al., 2020) and inclusion of BSF does not have adverse consequences for gut health or morphology even when it replaces all FM in

SW phase Atlantic salmon (Li et al., 2020) and at moderate (10%) and high (85%) inclusion levels in FW phase Atlantic salmon (Li et al., 2019). There is increasingly evidence that the presence of BSF may improve symptoms of SMBIE in salmonids (Kumar et al., 2021; Weththasinghe et al., 2021b). Insects including BSF contain chitin a component of the exoskeleton, a highly abundant mucopolysaccharide polymer of N-acetyl-d-glucosamine, (1–4)-linked 2-acetamido-2-deoxy-β-d-glucan (Henry et al., 2015; Park and Kim, 2010; Zarantoniello et al., 2020). It has been suggested that the presence of chitin is a limitation to using BSF for aquafeed inclusion, that it can cause reduced feed utilisation in Atlantic salmon and in small, may be pro-inflammatory (Lee et al., 2008; Olsen et al., 2006; Weththasinghe et al., 2021a).

Microbial ingredients from yeast sources have a long history in both human food and animal feed, and consequently have high consumer acceptance (Linder, 2019). Yeast species that have been considered in salmonid aquaculture research include, *Cyberlindnera jadinii* (anamorph *Candida utilis*), *Kluyvermoyces marxianus*, *Saccharomyces cerevisiae* and *Wickerhamomyces anomalus* (Agboola et al., 2020). They are approved for feed inclusion by the European Commission regulation No 68/2013 on a wide range of substrates (Lähteenmäki-Uutela et al., 2021; Øverland and Skrede, 2017). Yeasts have a crude protein content between 40-60% dependent on the species, strain and production method (Agboola et al., 2020; Øverland and Skrede, 2017). They have a similar amino acid composition to fish meal, except for methionine which is lower in yeasts (Alriksson et al., 2014; Øverland and Skrede, 2017).

Existing studies of yeast in Atlantic salmon diets suggest that up to 40% of torula yeast (*C. jadinii*) and *K. marxianus* can replace up to 40% of crude protein from fishmeal without adverse impact during the FW phase, however, *S. cerevisiae* at this inclusion level is poorer performing, reducing growth performance and nutrient utilisation (Øverland et al., 2013). Atlantic salmon fed a diet containing 25% of torula yeast from the FW through to the SW phase grew better than FM diets and had higher feed intake (Sahlmann et al., 2019). *C. jadinii* and *K. marxianus* have shown comparable digestibility to FM for Atlantic salmon, while *S. cerevisiae* had lower digestibility (Øverland et al., 2013; Sharma et al., 2018). Total digestion of yeast may be limited by the presence of nondigestible fractions in the cell wall (Sharma et al., 2018), however, processing of *S. cerevisiae* by autolysis improves digestibility (Agboola et al., 2021; Hansen et al., 2021). Yeast is palatable and leads to improved feed intake, thus increasing their suitability to be combined with plant proteins that can reduce feed intake (Sahlmann et al., 2019). Yeast also has other characteristics that make them well suited for farmed fish, such as positive effects on health, for example certain yeast species have been shown to counteract SBMIE in Atlantic salmon (Grammes et al., 2013; Sharma et al., 2018) and reduce inflammatory responses in post-smolts (Leclercq et al., 2020; Reveco-Urzua et al., 2019; Sahlmann et al., 2019).

1.2.3 Impact of protein source on growth performance

Growth and productivity are key indicators used to assess the performance of conventional and alternative proteins for salmon feed (Aas et al., 2019; Daniel, 2018). There is a trend that increasing inclusions levels of plant proteins such as SBM and SPC results in decreasing growth performance in salmonids (Collins et al., 2013; Turchini et al., 2019). Such differences in growth between FM and both SBM and SPC may be due to reduced digestibility of protein and energy, and for SBM, the presence of ANFs (Collins et al., 2013; Krogdahl et al., 2003). Growth performance is strongly linked to the type and inclusion level of alternative proteins. For BSF proteins in salmonid diets results suggest that 85% of dietary protein can be replaced with BSF in juvenile Atlantic salmon without significant reduction in growth , and 100% for post-smolt Atlantic salmon (Belghit et al., 2019). However, there are conflicting findings in the literature 25% replacement of conventional proteins with BSF meal is optimal for growth (Weththasinghe et al., 2021a), a consensus supported by other publications (Henry et al., 2015; Nash and Moore, 2020), no research has yet reported BSF improving growth performance compared to conventional proteins (Henry et al., 2015; Tran et al., 2015). In juvenile Rainbow trout (*O. mykiss*) the inclusion of up to 25% of yellow mealworm (*T. molitor*) replacing 100% of FM actually improved growth performance (Rema et al., 2019) although the general trend for insects in salmonid diets indicate that moderate inclusions are comparable to conventional proteins (Henry et al., 2015). For microbial proteins from yeast, *C. jadinii*, *K. maxianus* and *S. cerevisiae* showed some differences in growth performances for Atlantic salmon. For *C. jadinii*, feeding up to 25% (replacing convention proteins FM and SPC) from the FW phase through to the SW phase actually enhanced growth performance (Sahlmann et al., 2019). Furthermore when 40% of crude protein (FM) was substituted with *C. jadinii* in the FW stage, there was no significant impact to growth performance (Øverland et al., 2013), comparably in the same study *S. cerevisiae* reduced growth performance compared to the conventional protein diet, and final fish weight was also lower in *K.*

marxinus diets than the control. There is, therefore, a need to determine optimal inclusion levels of yeast to maximise Atlantic salmon growth.

1.2.4 Production and supply chain of alternative proteins

Insects are terrestrial invertebrates that are traditionally harvested from the wild for human consumption (van Huis, 2019), but as consumer and industry demand has increased, intensive insect farming has emerged (Cortes Ortiz et al., 2016) although the scale is still fairly small. Insect farming is needed to reduce pressure on wild habitats and populations, and to control the quality and availability of supply (van Huis, 2019). Insects are reared in environmentally controlled and sterile conditions, with species dependent nutritional requirements (Makkar et al., 2014), low water and land-use requirements, and can be reared at high density with low methane emissions, independent of location (Makkar et al., 2014; Rumpold and Schlüter, 2013; Van Huis and Dunkel, 2017). BSF larvae are usually harvested at instar stages 5 and 6 (the two stages prior to pupating (Kim et al., 2010)), when they have a high protein content, but before they reach the adult stage (Bosch et al., 2019). As omnivores, insects have become popular for their ability to grow on a wide range of substrates and because of their high feed conversion ratio making them very efficient bio-converters of low value organic matter to high value protein (Cortes Ortiz et al., 2016; Tinder et al., 2017). Research has explored a wide range of substrates including farm animal manure (Oonincx et al., 2015), plant material (e.g. potato, plantain and cabbage) (Parra Paz et al., 2015), spent grains (Kim, 2016), seaweed (Swinscoe et al., 2019), fisheries wastes (St-Hilaire et al., 2007) and organic waste from municipal sources (Diener et al., 2011). However, currently in Europe the use of substrates for farmed insects are limited to plant sources and use of manure and content from the digestive tract, processed animal proteins and catering waste for insects used in animal feed is prohibited (EU regulation 2017/893, EC regulation 178/2002, EC regulation 767/2009, EC regulation 1069/2009) (Bosch et al., 2019; Hua et al., 2019). Thus, selecting sustainable, safe and nutritionally suitable substrates is critical as life cycle analysis (LCA) of insects reared on food mixed with dried distillers grains actually had a greater environmental impact than conventional protein sources for aquaculture (Bosch et al., 2019), whereas insects reared on food by-products were 2-5 times more environmentally beneficial than conventional feeds (Smetana et al., 2016). **Figure 2** illustrates an example of the production supply chain for aquaculture using BSF.

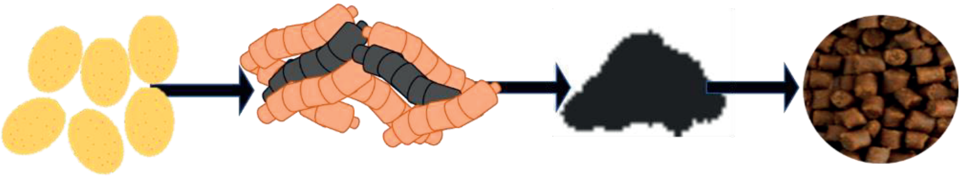

Figure 2. A linear supply chain for the insect, black soldier fly, reared on food waste, processed into meal, and used in formulated aquafeeds as a protein source.

Yeast are single-celled fungi and are most commonly batch-cultivated on sugar rich substrates under aerobic and low pH (4.5-5.0) conditions and moderate temperature (30°C) (Gómez-Pastor et al., 2011). Yeast has a long tradition of intensive cultivation for the production of bread, beer and more recently, ethanol and biodiesel, pigments, functional feed ingredients and as a protein source for animal feed (Malairuang et al., 2020; Marova et al., 2012; Øverland and Skrede, 2017; Selim and Reda, 2014; Subhash and Mohan, 2014). It can be produced independent of climate and season, and has a small environmental footprint (Couture et al., 2019; Suman et al., 2015). Yeast can be reared on beet molasses and cheese whey (Takakuwa and Saito, 2010), palm oil mill effluents (Saenge et al., 2011), olive mill waste water (Bellou et al., 2014; Dourou et al., 2016), banana peel hydrolysate (Jiru and Melku, 2018), lignocellulosic biomasses (Øverland and Skrede, 2017; Sharma et al., 2018; Solberg et al., 2021), and other agro-industrial waste streams(Leiva-Candia et al., 2014). Lignocellulosic biomasses have gained popularity in the research literature for bio-conversion to yeast for the aquaculture sector (Ahmadi et al., 2010; Muylle et al., 2015; Øverland and Skrede, 2017). They are comprised of hemicellulose (10-24%), cellulose (30-56%), lignin (3-30%), and low protein content (3-7.2%). They have the advantage of being a low-cost, highly available as a by-product from renewable sources (Ahmadi et al., 2010; Sindhu et al., 2016; Solberg et al., 2021). Lignocellulosic biomasses can be derived from wood, straw, seaweed, and perennial crops, much of which can be sourced from by-products of forestry or agriculture (Øverland and Skrede, 2017; Sindhu et al., 2016). These biomasses are enzymatically hydrolysed which produces pentose and hexose sugars which can then be utilised through aerobic fermentation by microorganisms, such as the yeasts, *C. jadinii* and *K. marxianus* which can simultaneously use these sugars (Kim et al., 2010; Øverland and Skrede, 2017; Solberg et al., 2021; Young et al., 2010). This supply chain is illustrated in **Figure 3** using the forestry sector as an example. This technology is still in its infancy and there are currently high costs associated with conversion of lignocellulosic biomasses to yeast and the volumes that are needed for the feed sector are not currently being produced (Agboola et al., 2020; Solberg et al., 2021).

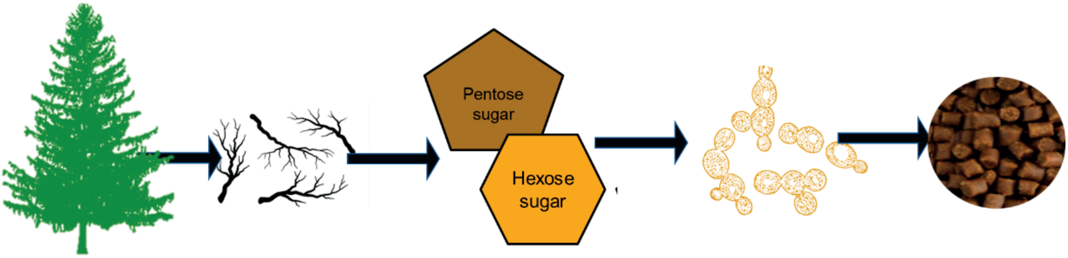

Figure 3. A linear supply chain for torula yeast cultivated on pentose and hexose sugars derived from forestry side streams of lignocellulosic biomass and used in formulated aquafeeds as a protein source

The availability of technologies and substrates from by-product sources for insect rearing and yeast cultivation will have an important role in sustainable supply chains and future food security as they have high potential to be incorporated into circular bioeconomies (Cappellozza et al., 2019; Grimm and Wösten, 2018). Circular economies are economic models that close the loops within industrial production and consumption systems (Ghisellini et al., 2016). They provide a method to use resources more efficiently and reduce the volume of waste produced (Regueiro et al., 2021). Bioeconomies are those economies that are driven by innovations in biotechnology and production of renewable resources along with their waste streams that can be re-valorised through strong cross-sector interaction and facilitated by circularity (Carus and Dammer, 2018; Vieira et al., 2020). There is currently strong political attention around the benefits of circular economies and circular bioeconomies for sustainable development, with European Commission initiatives such as, the Circular Economy Action Plan which is a central part of the 2019 European Green Deal and aims to make Europe an efficient resource user with a reduced consumption footprint (Cecchin et al., 2021; Siddi, 2020). The United Nations Sustainable Development Goals (SDGs) also have an number of targets that require the development and promotion of the circular economy (Schroeder et al., 2019). There is a growing body of research that considers the potential of aquaculture in circular bioeconomy models, with focus on improving the sustainability of production, ensuring the resilience of the supply chain and reducing waste by using resources more efficiently (Hamam et al., 2021; Regueiro et al., 2021). Such models will be particularly important in the commercial uptake of alternative and novel feed proteins for the aquaculture sector (Kusumowardani and Tjahjono, 2020; Ritala et al., 2017). Insects and yeast proteins can both be grown on such re-valorised side streams from other industries (Liguori and Faraco, 2016; Ojha et al., 2020; Øverland and Skrede, 2017; Parra Paz et al., 2015). Yet, knowledge gaps exist for the suitability alternative protein grown on varied substrate for aquafeed inclusion. Such a non-linear model could then be combined with technological innovations that allow for utilisation of farmed fish by-products, such as Atlantic salmon skin collagen (Coppola et al., 2021), and salmon waste for agricultural fertiliser (Celis Hidalgo et al., 2008) which could in turn support the growth of crops with high lignocellulosic content such as perennial grasses (Muylle et al., 2015) in a closed-loop example illustrated in **Figure 4**. Such a model would have an additional benefit for the agro and aquaculture sectors, allowing currently under-utilised biomasses like seaweeds, perennial grasses, and legumes in Europe to be sustainably exploited either indirectly or directly for the feed sector (Kostas et al., 2021; Rafoss and Nagothu, 2020). This would further reduce the European protein deficient (Smárason et al., 2019) and could enhance the value of ecosystem services (ES) provided by European production systems (Thomas et al., 2021) creating a positive feedback loop. However, there exist knowledge gaps for how such a supply chain might be implemented for aquaculture and the impact to environmental health.

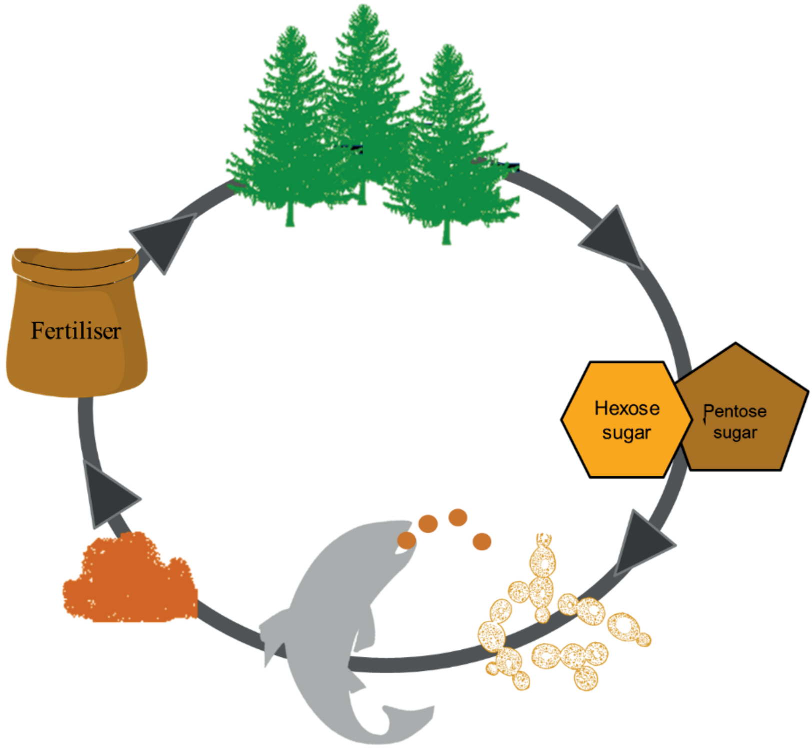

Figure 4. A conceptual closed loop circular bioeconomy model for Atlantic salmon aquaculture. Yeast is cultivated on lignocellulosic biomasses from forestry, fed in formulated aquafeeds to Atlantic salmon and waste from aquaculture is processed into fertiliser to support sustainable forestry productivity.

1.2.5 Processing and addition of functional ingredients

To optimise ingredients and diets for aquaculture, research has explored methods of processing and supplementing conventional and alternative ingredients. SBM, in particular, has received such research attention to improve its value for salmonid feeds (Bedford, 2018; Chen et al., 2013; Drew et al., 2007). Targeted processing to reduce ANFs in SBM include solvent extraction and treatment with exogenous enzymes (Drew et al., 2007) which can limit gut inflammation (Green et al., 2013). Enzymes such as β glucanase, xylanase, phytase, and cellulase (Kirk et al., 2002; Li et al., 2020) can break down the bonds in the NSPs, reducing them to oligosaccharides, and can bio-transform saponins (Jacobsen et al., 2018). Existing research has also tried to improve the application of SBM through the fermentation (chemical breakdown by microorganisms) for example with the yeast *S. cerevisiae*, and bacteria *Lactobacillus bulgaricus,* and *Bacillus subtilis* (Chen et al., 2013; Li et al., 2020; Mukherjee et al., 2016). Fermentation has also been applied to fisheries side streams (Mondal et al., 2008), insect proteins (Poelaert et al., 2016), and green crops (Santamaría-Fernández et al., 2017) to improve nutritional quality, digestibility, increase protein content and even improve shelf-life of these ingredients for use in monogastric feeds (Catalán et al., 2018; Dawood and Koshio, 2020; Hong et al., 2004; Klunder et al., 2012; Refstie et al., 2005). It has been suggested that processing BSF by dechitination (removal of chitin) may improve its application to salmonid diets (Weththasinghe et al., 2021a) although some literature suggest chitin may have benefits for fish gut health (Askarian et al., 2012; Zhou et al., 2013).

Functional feed additives are gaining research attention for aquaculture to improve compatibility of feed ingredients, to stimulate immune resilience, and improve health and growth performance (Encarnação, 2016). In salmonid aquaculture, prebiotics and probiotics have gathered traction and are increasingly used commercially. Prebiotics are defined as non-digestible components of feed that stimulate the proliferation and/or activity of bacteria residing in the gut (Ganguly et al., 2013). Prebiotics have been added to feeds to improve production and health of terrestrial farmed animals but have only recently gained attention in the aquaculture research literature (Ringø et al., 2010). Primarily these are small fragments of carbohydrates, and small sugars such as galactose and mannan-oligosaccharides commercially known as MOS, β-glucan and fructooligosaccharide (FOS) added as immune-stimulants in aquaculture (Ganguly et al., 2013; Mohan et al., 2019; Song et al., 2014). Prebiotics like β-glucanase and enzymes have also been added to the diets of pigs (Yin et al., 2001) and broiler chickens (Campbell and Bedford, 2011) to access greater nutritive value of high NSP ingredients, such as hulled barely. Probiotics are live bacteria added to feeds, that change conditions in the fish gut (Wang et al., 2008). In Atlantic salmon, the probiotic bacteria, *Pediococcus acidilactici* has been shown to modulate the gut microbiome and improve gut health by reducing inflammation (Jaramillo-Torres et al., 2019; Vasanth et al., 2015). Lactic acid bacteria (LAB) a group of bacteria considered beneficial for finfish and crustaceans (Ringø et al., 2020) have been added to commercial feeds (Balcázar et al., 2006), SBM (Navarrete et al., 2013; Ringø et al., 2020), and insect proteins for the freshwater crayfish (*Chera cainii*)(Foysal et al., 2021). Yet, the is a lack of information on the optimal processing methods for existing and potential aquafeed proteins for Atlantic salmon.

1.2.6 Feed and the gut microbiome

The gastro-intestinal (GI) tract of animals is inherently, directly impacted by the composition of feed. Aquaculture research has highlighted the importance of gut health in the resilience of the innate immune system of fish, which is an organisms first line of defence and for the adaptive immune system which is an organisms specialised defence (Gómez and Balcázar, 2008; López Nadal et al., 2020; Martin and Król, 2017). Feed ingredients can alter the microbial ecology of the GI (Daniel, 2020; Hans-Christian Ingerslev et al., 2014; Perry et al., 2020). The gut microbiome refers to the microbial flora present in GI tract that has a strong influence on many aspects of host survival and development, affecting growth performance (Perry et al., 2020), disease resistance and immune function (Gajardo et al., 2016; López Nadal et al., 2020; Yukgehnaish et al., 2020), access to and uptake of nutrients that would not otherwise be accessible to the host (Ghanbari et al., 2015; Llewellyn et al., 2014; Merrifield and Rodiles, 2015), metabolism (Dvergedal et al., 2020), inflammation of the gut (Kumar Issac et al., 2020), and behaviour (Soares et al., 2019). In turn, the composition of the gut microbiome of fish is influenced by the host species (Yukgehnaish et al., 2020), life stage (Llewellyn et al., 2016; Michl et al., 2019), sampling approach and section of the gut (Gajardo et al., 2017), the surrounding environment, and whether the host is wild or farmed (Dehler et al., 2017a), genetic history (Smith et al., 2015), the health and welfare status of the host (Webster et al., 2020), feeding behaviour (Talwar et al., 2018), and feed composition, including the protein type and source (Gajardo et al., 2017; Michl et al., 2019). The FW stage of farmed salmon are dominated by the phyla, *Proteobacteria* (mostly *Escherichia*) followed by *Firmicutes* (mostly *Lactobacillus*) and *Actinobacteria* (all Other *Gp4*). There are significant differences after seawater transfer, the same fish were dominated by *Proteobacteria* (mostly *Esherichia)*, *Firmicutes* (mostly *Lactbacillus)*, *Bacteriodetes* (all *Prevotella*), and *Tenericutes* (all *Mycoplasma)* although some core microbiota is maintained across life stages in healthy individuals (Dehler et al., 2017b; Wang et al., 2021). Certain bacteria are associated with positive benefits for fish, including LABs (e.g. *Lactobacillus, Lactococcus ,Leuconostoc, Enterococcus, Streptococcus, Carnobacterium, Weisella and Pediococcus*), and *bacilli* (Ringø et al., 2020, 2018). They have been associated with improved digestive function, disease resistance (e.g. producing secondary metabolites that inhibit pathogen growth (De Vuyst and Leroy, 2007), and improved gut health. In Rainbow trout, specific groups of microbes have even been associated with fast growing (*Clostridium*, *Leptotrichia*, and *Peptostreptococcus*) and slow growing individuals (*Corynebacterium* and *Paeniclostridium*) (Chapagain et al., 2019). Comparatively, the gut microbiome community, can become imbalanced and can lead to negative impacts on the host immune system through loss of desirable bacteria or presence of pathogens, and can reduce aquaculture productivity, a state known as dysbiosis (Infante-Villamil et al., 2021; Perry et al., 2020; Wang et al., 2018). It should be noted, that there is a large variation between studies, methods, individuals (Green et al., 2013), environment, between wild or farmed hosts (Llewellyn et al., 2016), and between different culture systems, such as Recirculating Aquaculture Systems (RAS) or sea cage farms (Dehler et al., 2017a) which creates challenges when drawing conclusions from the existing literature.

Research has characterised the Atlantic salmon gut microbiome fed a range of different proteins. However, there are substantial knowledge gaps characterising the impact to microbiome during early life stages, and the microbiome consequences of different processing methods for proteins (Hartviksen et al., 2014). This is especially important for juvenile salmonids as early feeding and feed strongly influence the initial establishment of their gut microbiome (Especially in the digesta (Gajardo et al., 2017; Li et al., 2021)) (Rodríguez et al., 2015; Siriyappagouder et al., 2018). The early gut microbiome of salmonids is highly malleable and strongly influenced by dietary composition (Michl et al., 2017, 2019) and subsequently shaped by the environment (Dehler et al., 2017a; H.-C. Ingerslev et al., 2014; Michl et al., 2017, 2019).

When SBM is included in salmonid diets, significant gut microbiome changes have been detected compared to FM, for example increased presence of lactic acid bacteria (LAB), normally associated with benefits for the host and likely driven by increased indigestible fibres present in SBM, but benefits may be limited by the presence of SBMIE (Gajardo et al., 2017). When SBM has been fermented with LAB, the presence of LAB in the gut was increased and potential benefits for host intestinal health were detected (Catalán et al., 2018). For SPC , an increase in gut microbiome diversity was also detected, influenced by non-indigenous bacteria that are not normally present and introduce potential risks, such as bacillary dysentery and altered intestinal salt and water balance, even in the absence of detectable morphological change (Green et al., 2013). Furthermore, addition of immuno-stimulants like MOS ameliorate this impact. However, not all studies found such differences between SPC compared to FM in Atlantic salmon (Hartviksen et al., 2014). Other plant proteins have similarly shown alteration to the gut microbiome of salmonids particularly during early development (Michl et al., 2017).

The use of insect proteins has been less widely studies for salmonids diets, however, existing evidence shows increased alpha diversity, presence of LABs and abundance of *Firmicutes* in BSF fed Rainbow trout (Bruni et al., 2020; Huyben et al., 2019). In seawater the microbiome community richness and diversity was increased in Atlantic salmon fed BSF diets (Li et al., 2020), but the impact of BSF during the freshwater stage has not been characterised and represents a substantial knowledge gap. The presence of chitin in insect proteins provides an interesting debate in the salmonid aquafeed literature, since it has both been added as a functional feed supplement that promoted microbiome diversity and presence of LABs (Askarian et al., 2012), but also it has been suggested to be problematic for feed utilisation, and removal has been recommended (Olsen et al., 2006; Weththasinghe et al., 2021a). To optimise the inclusion of insects in diets, it will be important to elucidate the impact of insects on the gut microbiome and how processing alters impact.

Diets formulated with yeast proteins for salmonids have shown greater impact on the gut microbiome at higher inclusions levels, for example for *S. cerevisiae* and *W. anomalus*, which altered the bacterial community for Rainbow trout at 40% and 60% but not at 20% inclusion compared to FM diets (Huyben et al., 2017). Despite the increasing interest in the *C. jadinii* yeast as an alternative protein source, the impact on the salmonid gut microbiome has not yet been characterised and represents a significant knowledge gap. In pigs *C. jadinii* has been associated with selection for the LAB *Lactobacillus spp*. in the gut microbiome (Iakhno et al., 2020).

1.2.7 Feed and behaviour in fish

Recent research has identified a bi-directional link between the composition of the gut microbiome in animals, including fish, on the brain and consequently behaviour which has be dubbed the microbiota-gut-brain-axis or gut-brain-axis (Foster et al., 2017; Martin and Mayer, 2017). Research for the gut-brain-axis rose to prominence in the field of human biomedical science, with links between mental health, eating habits and even autism and the gut microbiome, particularly linked to the presence of dysbiosis and gut inflammation (Clapp et al., 2017; Martin and Mayer, 2017). The two-way link between the microbiota in the GI tract and the brain is a consequence of secondary metabolites and neurochemicals produced by the microbiota in the gut, and neurotransmitters from the host that influence the establishment and composition of the gut community (Kraimi et al., 2019). For example, in Zebrafish (*Danio rerio*), caffeine, a drug that causes neural hyperactivity, also disordered the gut microbiome of fish, but exposure to melatonin restored a normal microbiome community (Zhang et al., 2021). Since feed ingredients alter the composition of the gut microbiome, an impact to behaviour and consequently welfare is possible (Ibarra-Zatarain et al., 2015; Kraimi et al., 2019).

Behavioural measurements have become an increasingly important tool to measure and monitor farmed Atlantic salmon, both for environmental and economic optimisation such as feeding and food delivery to cages (Føre et al., 2011; Li et al., 2020), and to optimise animal welfare (Barreto et al., 2021; Brijs et al., 2021). Welfare of farmed fish has gathered increasing attention from the scientific community (Huntingford and Kadri, 2014) and from consumers (Solgaard and Yang, 2011). Furthermore, good fish welfare has been associated with better health and productivity for fish, and better quality product for consumers, making it a priority for the aquaculture industry (Benhaïm et al., 2009; Poli, 2016). Good welfare is defined as the absence of suffering and conditions allowing the five freedoms (freedom from thirst, hunger and malnutrition, freedom from discomfort, freedom from pain, injury and disease, freedom to express normal behaviour, and freedom from fear and distress) (Huntingford et al., 2006; Webster, 2001)

Traditional measures of behaviour in finfish aquaculture include fin-nipping and aggression, swimming activity and changes in feeding behaviour (Martins, Catarina, I et al., 2012). Increasingly, the importance of animal personality and coping style have gained momentum for detecting early warning signs of impact from changing environmental or physiological conditions in aquaculture, such as inducing triploidy (Benhaïm et al., 2020), change to feeding or environment (Castanheira et al., 2015) , or changes in dietary composition (Ibarra-Zatarain et al., 2015). Animal personality or coping style define the individual characteristics that are consistent over both time and situation (Benhaïm et al., 2020; Koolhaas et al., 1999). These measures are particularly relevant for intensive aquaculture since they indicate how adaptable a fish may be to changing conditions and how susceptible to stress-related disease (Castanheira et al., 2015; Huntingford and Adams, 2005; Koolhaas et al., 1999). Coping style separates individuals into proactive or reactive strategies based on behavioural responses and neuroendocrine responses to challenging conditions (Koolhaas et al., 2010, 1999). In fish research, there has been increasing interest in non-invasive measurements of behaviour responses to characterise coping style

(Benhaïm et al., 2020; Skov et al., 2019). **Table 1** shows behavioural responses associated with the two different styles adapted from (Castanheira et al., 2015). Proactive individuals are those that are faster to explore their environment, bolder, more likely to take risks, show more socially dominant behaviour and have low sensitivity to stress. Reactive individuals are those that are cautious and avoid risk, show lower tendency to explore, are shyer, and socially submissive, but have high response to environmental change (Benhaïm et al., 2020; Castanheira et al., 2015). Individual behaviour falls on a continuum between these two styles (Benhaïm et al., 2020). Intermediate individuals are suggested by some research to be those with the best growth performance (Skov et al., 2019) although this finding is not consistent across the literature, with proactive salmon also recorded as faster growers (Damsgård et al., 2019). Proactive styles have also been associated with high reproductive success in farmed gilthead seabream (*Sparus aurata*) (Ibarra-Zatarain et al., 2019). Selective breeding programs for intensively farmed animals like Atlantic salmon, may have selected for certain coping styles (since coping style is heritable (Ruiz-Gomez et al., 2008)), inadvertently promoting proactive individuals that are more stress-tolerant, and this may have had unintended consequences, creating selection pressure on the gut-brain-axis and favouring certain gut microbiome communities (Cairo et al., 2021; Milla et al., 2021). Research has been initiated for European seabass (*Dicentrarchus labrax*) aiming to investigate the potential for developing selective breeding programs targeting personality traits (Ferrari et al., 2016). Environmental stressors that influence fish at juvenile development stages are especially important to measure as they may create selection pressure that shapes the behavioural characteristics later in life, selecting for more adaptable traits (Hope et al., 2020; Nederhof and Schmidt, 2012) and affecting social hierarchy (Ruiz-Gomez et al., 2008). In the wild, both proactive and reactive strategies exist and have benefits and trade-off for individual survival and fitness (Ruiz-Gomez et al., 2011). Yet, in farm conditions the coping style may influence the quality of life, and welfare experienced by intensively cultivated farmed salmon (Finkemeier et al., 2018; Johansen et al., 2020; Veissier et al., 2012). More proactive styles have lower sensitivity to environmental stress, quicker recovery from stresses like transport, and may be traits that are beneficial to the welfare and production of domesticated animals and thus, favoured by artificial selection, however the flexibility of reactive fish may support them being more resilient to novel stressors (Huntingford and Adams, 2005; Koolhaas, M and van Reenen, G, 2016). When there is a correlation between behavioural traits across time and context, this is referred to as a behavioural syndrome, which has implications for fitness (Sih et al., 2004). Further research is needed to elucidate the welfare status under differing coping styles for Atlantic salmon to optimise behaviour measures for intensive aquaculture.

Nutritional composition of feed can have consequences for behaviour and specifically for coping style. In juvenile Senegalese sole (*Solea senegalensis*) found individuals fed fish oil were more proactive than individuals fed plant oil (Ibarra-Zatarain et al., 2015). Juvenile Rainbow trout fed a plant-based diet displayed increased apathetic behaviour and an elevated stress response (Sadoul et al., 2016), characteristics of more reactive individuals. When diets of larval yellowtail (*Seriola quinqueradiata*) were enriched with DHA and EPA, these individuals shoaled more tightly compared with groups enriched with oleic acid (an omega-9 fatty acid), where shoals were more dispersed (Ishizaki et al., 2001). In seabream fed diets enriched with long-chain poly-unsaturated fatty acids (PUFA), proactive behaviours were increased, decreasing response time to a stress stimulus (Benítez-Santana et al., 2012). There are a growing number of novel cross-discipline studies that are exploring the link between dietary composition, gut microbiome and coping style that support the need to explore all these aspects to optimise aquafeeds. In Zebrafish, the probiotic *Lactobacillus rhamnosus* IMC 501, altered the gut microbiome, brain gene expression and altered shoaling behaviour (Borrelli et al., 2016). Links have been found in the mammal species, collared peccary (*Pecari tajacu*), between dietary treatments, gut microbiome composition and coping style, that suggest more evenly composed bacterial communities were present in calmer (proactive) individuals (Cairo et al., 2021). Such dietary manipulation could prove highly valuable for stress-related welfare in farm conditions and highlights the importance of assessing impact of dietary composition on behaviour in addition to gut microbiome and traditional production measures. There are large knowledge gaps for the impact of fish meal and soy derived proteins on coping style and consequently welfare for Atlantic salmon which must be resolved to optimise the application of conventional proteins, and subsequently this knowledge gap must be closed for alternative proteins before they are implemented in formulated aquafeeds. A conceptual diagram is provided in **Figure 5** to visualise the importance of this relationship for Atlantic salmon feed ingredients on the gut-brain axis in aquaculture.

Table 1. The behavioural responses of reactive and proactive coping styles adapted from (Koolhaas et al., 1999; Maria Filipa et al., 2015).

Response	Proactive style	Reactive style	Reference
Feed efficiency	high	low	(van de Nieuwegiessen et al., 2008)
Time to feed post-stress	low	high	(Ruiz-Gomez et al., 2008; Vaz-Serrano et al., 2011)
Feeding motivation	high	low	(Silva et al., 2010)
Exploration & Risk taking	high	low	(Skov et al., 2019)
Aggressiveness	high	low	(Vaz-Serrano et al., 2011)
Sensitivity to environmental stressors	low	high	(Höglund et al., 2008; Catarina I. M. Martins et al., 2011)
Plasticity	low	high	(Ruiz-Gomez et al., 2011)
Routine formation	low	high	(Mesquita et al., 2015)
Active avoidance of stressor	high	low	(Damsgård et al., 2019; Catarina I.M. Martins et al., 2011)

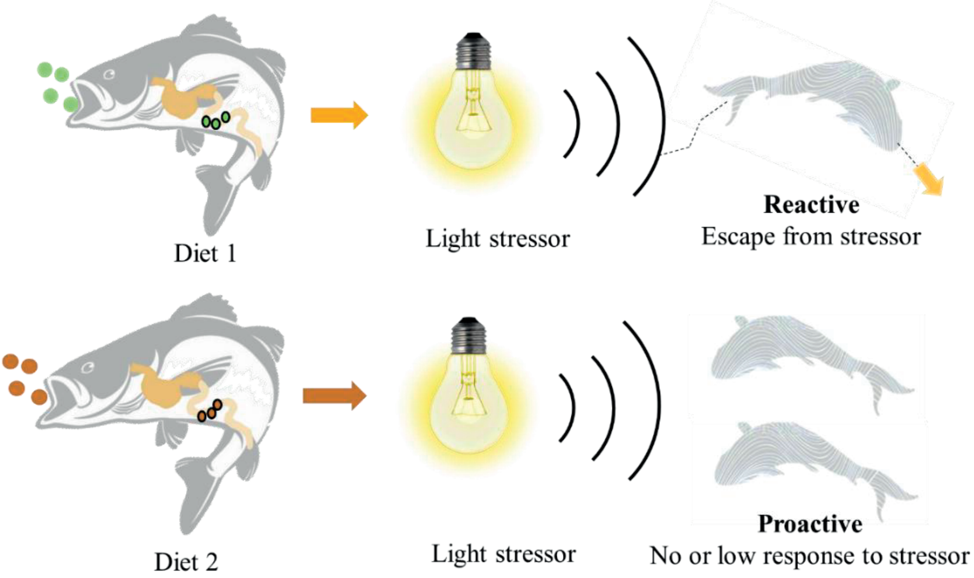

Figure 5. Conceptual diagram of how bi-directional signalling between the gut and the brain (gut-brain-axis) could be influenced by changing the protein source in diets fed to Atlantic salmon. The change in diet may alter the gut microbiome and thus, produce differing behavioural traits or coping styles in fish. In this example, the plant-based diet (top) influences greater sensitivity to a light stressor and, thus a more reactive individual, whereas the marine-based diet (bottom) influences greater tolerance to a light stressor and, thus a more proactive individual.

1.3 Aims, hypothesis, and objectives

The aim of this thesis was to address three research priorities identified from the existing literature that would support long-term improvement of the Atlantic salmon aquaculture sector (**Figure 6**). The first priority was to characterise and optimise the proteins that are currently available to the aquafeed sector, the second priority was to characterise and optimise alternative proteins that have potential for the aquafeed sector, and the third priority was to investigate mechanisms for sustainable exploitation of new protein sources. The global hypothesis of this thesis was that addressing these priorities will benefit growth, health, welfare, and environmental impact of Atlantic salmon aquaculture compared to using conventional proteins.

To test this global hypothesis, three multi-disciplinary experiments and a literature review were performed with the following specific objectives:

1. To optimise the use of SBM and characterise the impact of novel SBM enhancements on growth performance, gut microbiome, and behaviour relevant to welfare in aquaculture (*Paper I*).
2. To optimise the use of BSF reared on food waste and characterise the impact of novel processing of BSF on growth performance, gut microbiome, and gut health (*Paper II*).
3. To optimise the inclusion of torula yeast cultured on lignocellulosic biomass and characterise the impact of torula yeast inclusion and combination with conventional proteins on growth performance and gut microbiome (*Paper III*).
4. To review the ecosystem services provided by under-utilised green and blue production systems, and their current and future role in the circular bioeconomy to supply ingredients to the aquafeed industry (*Paper IV*).

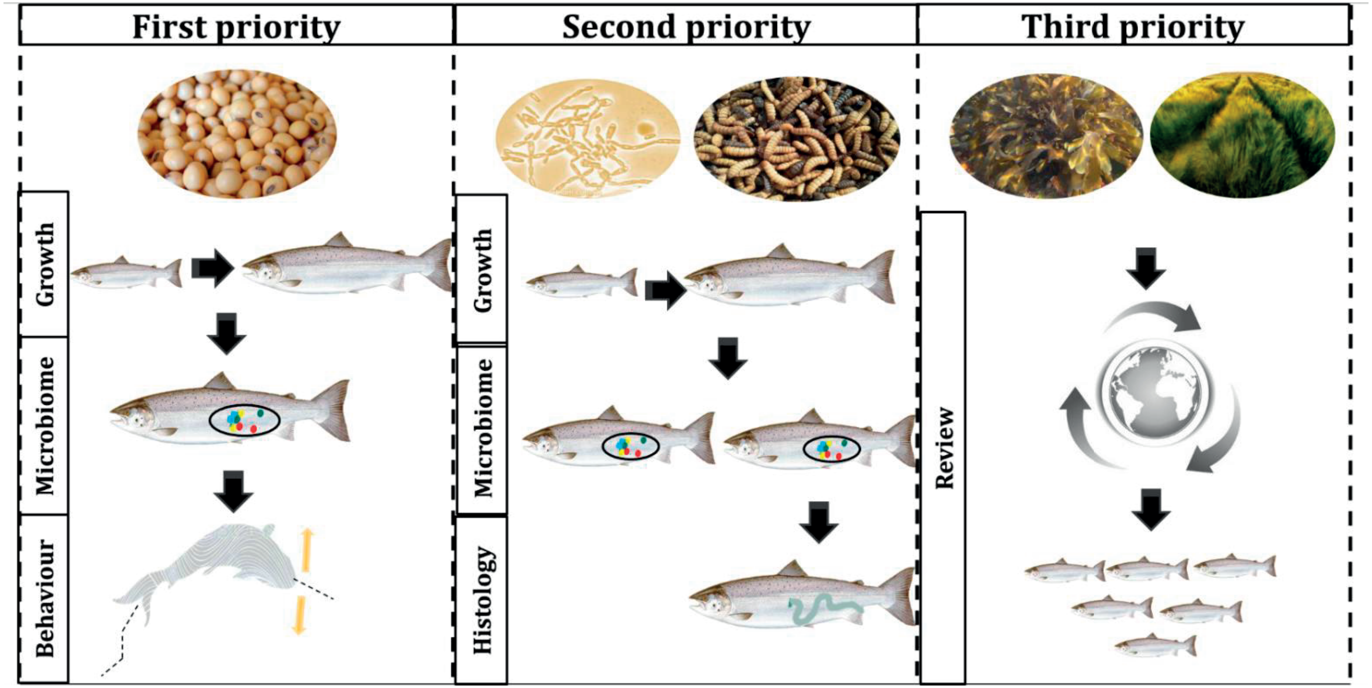

Figure 6. Overview of the three priorities that this thesis aims to address. The first priority, to optimise and characterise the proteins that are currently available to the aquafeed sector (*Paper I*), the second priority, to characterise and optimise alternative proteins that have potential for the aquafeed sector (*Paper II* and *III*), and the third priority, to review the mechanism for sustainable exploitation of new protein sources (*Paper IV*). These research priorities were address using a multi-disciplinary approach.

2 Materials and methods

This thesis used three methodologies that were either adapted or specifically developed to answer the research objectives of the studies included herein. These three methodologies are described in the following section for clarity.

2.1 Gut histology methodology

In *Paper II*, a gut histology methodology adapted from (Barnes et al., 2014; Colburn et al., 2012; Knudsen et al., 2007) was used to investigate the impact of processed and non-processed BSF compared to conventional proteins on the gut health of juvenile Atlantic salmon. A standard histological sample preparation was applied. Distal gut tissues were equilibrated in xylene and embedded in paraffin wax. Sections of 4μm thickness were cut and stained with hematoxylin and eosin (H&E). The sections were cut longitudinally (perpendicular to the folds) as expert advice determined this was the most effective way to set very small tissue samples from the gut. Processing was carried out at the veterinary department of The Norwegian University of Life Sciences (NMBU). Since the fish in this experiment were at an early development stage with an immature gut, the samples were very challenging to work with, and standard histological interpretation methods enabled just two variables to be reliably scored, the lamina propria of simple folds and the connective tissue between the base of folds and stratum compactum and a semi-quantitative scoring scale from 1-3 was selected to reflect the FW developmental stage and the condition seen across all samples **Table 2.** Blind histology examination was performed on images taken of slides under a light microscope with camera (Olympus BX51, Tokyo, Japan) with a magnification of (x100) (**Figure 7)**. Standard statistical approaches described in *Paper II* were then followed.

Table 2. Histological scoring system used on Altantic salmon fed experimental feed treatments in *Paper II* adapted from (Barnes et al., 2014; Colburn et al., 2012; Knudsen et al., 2007).

Score	Appearance
	Lamina propria of simple folds
1	Thin and delicate core of connective tissue in all simple folds.
2	Lamina propria slightly more distinct and robust in some of the folds.
3	Clear increase in lamina propria in most of the simple folds
	Connective tissue between base of folds and stratum compactum
1	Very thin layer of connective tissue between base of folds and stratum compactum.
2	Slightly increased amount of connective tissue beneath some of the mucosal folds.
3	Clear increase of connective tissue beneath most of the mucosal folds

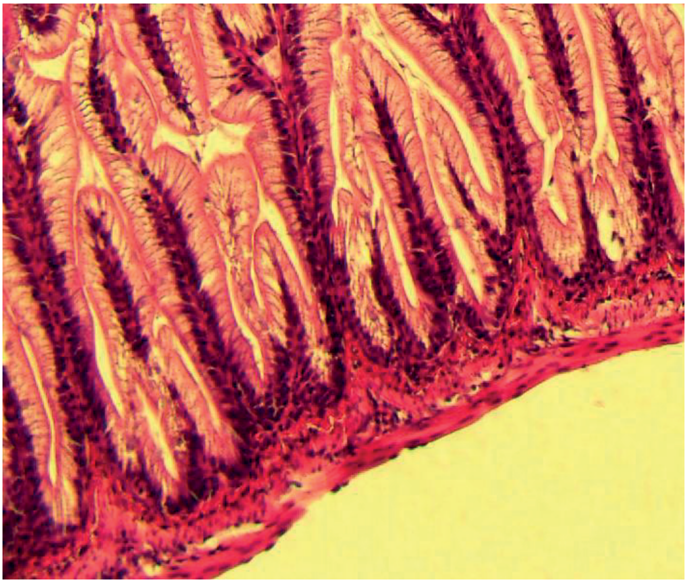

Figure 7. Histological section from FW Atlantic salmon taken from blindly scored fish in *Paper II*.

2.2 Gut microbiome methodology

In *Paper I*, *Paper II*, and *Paper III,* the gut microbiome of FW Atlantic salmon was analysed. Working with juvenile salmonids presents a challenge for DNA extraction and for DNA amplification. A novel methodology was developed during this PhD by students and researchers at Matís ohf, Iceland, to address these challenges. There were slight sampling differences, thus the first steps of downstream processing differed between *Paper II,* and *Paper I* and *Paper III*. This was due to slightly larger fish in *Paper II* and that these were the first samples collected in this doctorate. Further collection followed a newly standardised procedure that was developed for juvenile salmonids. Both methods were effective and are described.

In *Paper I* and *Paper III*, samples of the lower GI tract from the end of the pyloric caeca to the end of the distal gut, were sampled with the digesta and mucosa present under sterile conditions, following a 12-hour fasting period. Samples were stored at -80°C prior to downstream processing. GI tract samples were individually manually homogenised in a sterile petri dish with a sterile scalpel to physically break up the tissue. Sample material was transferred to a sterile 2ml Eppendorf tube, with 300μl of sterile 1mm diameter silica beads (BioSpec Products, United States) and 800μl of CD1 solution from the QIAamp PowerFecal Pro DNA kit (QIAGEN, Germany). Samples were vortexed for 5 seconds and shaken at maximum speed (30Hz) in a laboratory mixer mill (Retsch MM400) for 1 minute. This extra stage was added to help further break up the bacterial cells and maximise the concentration of DNA extracted. The supernatant (~800μl) was transferred to the PowerBead Pro tube from the QIAGEN QIAamp PowerFecal Pro DNA kit. The protocol for this DNA extraction kit was then followed exactly and final DNA was eluted with 80μl of C6 solution (**Figure 8**). A DNA negative (no material added) was also run to ensure no contamination occurred during DNA extraction. In *Paper II,* samples of the digesta from the distal gut of FW Atlantic salmon were sampled under sterile conditions after a 2-hour fasting period. Samples were stored at -80°C prior to downstream processing. Digesta samples were then transferred to the sterile 2ml Eppendorf tube with 300μl of sterile 1mm diameter sterile silica beads. After which the procedure was identical to that described for *Paper I* and *Paper III* (**Figure 8**).

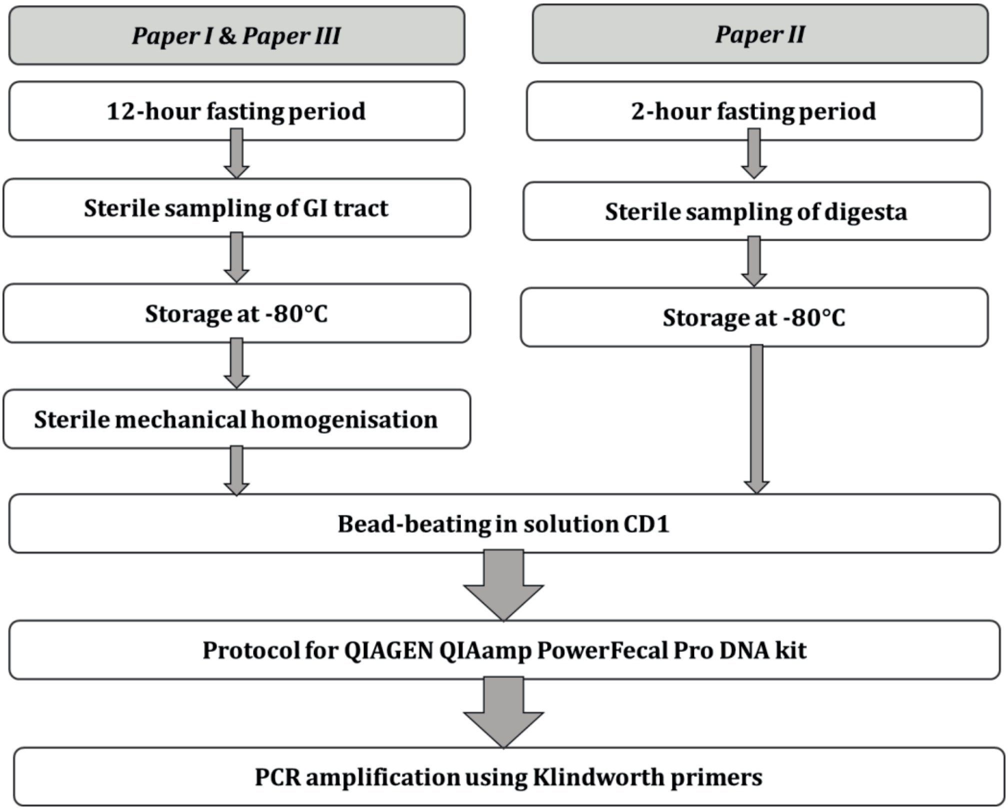

Figure 8. process of gut microbiome sampling in Atlantic salmon in *Paper I*, *Paper II* and *Paper III*.

For all three papers, an identical DNA amplification methodology was followed that was developed through trial and error and expert knowledge to maximise the DNA amplified. Samples were subjected to PCR of the V3-V4 regions of the 16S rRNA gene with a universal bacterial primer pair S-D-Bact-0341-b-S-17 (5′–CCTACGGGNGGCWGCAG-3′)/S-D-Bact-0785-a-A-21(5′-GACT-ACHVGGGTATCTAATCC-3′) (Klindworth et al., 2013). PCR master mix included diluted DNA, nuclease-free water, Q5 High Fidelity DNA polymerase (New England Biolabs, Ipswich, USA), Q5 GC Enhancer, 0.5μM of each primer containing Illumina overhand adapters, and 1 x Q5 Reaction buffer, 200μM dNTPs (New England Biolabs, Ipswich, USA). Both positive and negative samples were also run in the PCR to monitor for absence of contamination and successful amplification. The thermocycling protocol had an initial denaturation step (90°C for 30s), followed by 35 cycles of: denaturation (90°C for 10s), annealing (52°C for 30s), and extension step (72°C for 30s), with a final extension (72°C for 2 min). Libraries were multiplexed with Nextera XT v2 barcodes (Illumina, USA), normalised using Sequel-Prep normalisation places (ThermoFisher Scientific, USA) and sequenced on MiSeq desktop sequencer (Illumina, USA) using v3 chemistry and 2 x 300 cycles. Standard bioinformatic and statistical procedures were then followed and described in all papers.

2.3 Behaviour methodology

In *Paper I*, a specific combination of behavioural observations was carried out to assess the behavioural traits of boldness and exploration, and to assess the response to a stressor. These observations were carried out at the individual level through two specifically designed tests, a swimming activity test with a stressor, and an open field test with a shelter. Individuals were tracked through two replicates of these two different tests; thus, each individual was observed a total of four times, twice in two different tests. Coping style, the behaviour of an individual in challenging conditions (e.g. a novel environment) that is consistent over time and context could then be assessed to determine how individuals ranged along a proactive-reactive axis, and what is the impact of different feed treatments evaluated in *Paper I* (Koolhaas et al., 1999). Individuals were followed by unique colour combinations of visual implant elastomer (VIE) tagging below the dorsal fin, a low-impact method effective to follow juvenile fish (Leblanc and Noakes, 2012). All behaviour observations were carried out in a dedicated room and in purpose-built behaviour arenas that were lit from below. Individual behaviour was tracked using video recording and analysis with EthoVision XT software (Noldus, The Netherlands).

2.3.1 Swimming activity with stressor

The swimming activity test with stressor was used to collect data on the behaviour trait of exploration, and to determine the response to an elicited stress. This protocol was developed following Benhaïm et al. (2020). The elicited stress was to switch off the light below the arenas for 3 seconds and then switch it back on again. For the swimming activity test there were 4 circular arenas (diameter = 25cm, water depth 7.8cm (4L), height= 15cm) (**Figure 9)**. Tagged fish from each tank were collected from the trial tank and transferred in identical white transfer buckets with closed tops and then placed into individual arenas in random order. All four arenas were filmed simultaneous and continuously for 20 minutes and for the purposes of analysis was separated into four virtual periods. The 0-5 minutes was the acclimation period (AC), 5-10 minutes was the normal swimming period (NS). At the 10-minute mark a stress was elicited, minutes 10-15 was thus the post light stress period (PLS), and 15-20 minutes was the recovery period (RC). Variables collected through the swimming tests were, mean distance from centre-point (cm): the distance of the centre-point of the fish body from arena centre. Total distance moved (cm): the distance travelled by a fish measured from the centre-point of body between consecutive X-Y coordinates. Mean velocity (BL s^{-1}): the distance moved by the centre-point of body per unit time between consecutive X-Y coordinates normalised to body lengths per second. Absolute angular velocity (° s^{-1}): expressed in degrees per second was calculated by EthoVision XT software. Vangn = RTAn/tn – (tn-1), where RTAn represents the relative turn angle for sample n, and tn-(tn-1) is the time difference between the current and previous sample. Mobility state, the cumulative duration for which the fish body is changing, highly mobile (s) when cumulative duration is 60%. Moderate mobile (s) when cumulative duration was between 20-60%. Immobile (s) when cumulative duration was below 20%. Standard statistically techniques were followed and described in *Paper I*, assessing response to a stressor, and the trait of exploration, which was assessed using the NS period (5-10minutes) to assess the trait of exploration.

Figure 9. Swimming test arena used for observation of the behavioural trait of exploration and individual response to an elicited light stress.

2.3.2 Open Field Test with shelter

The Open Field Test (OFT) with shelter was used to collect data on the behaviour trait of boldness following (Benhaïm et al., 2020; Toms et al., 2010). For the OFT with shelter, 4 rectangular arenas (40 x 30 x 25 cm), water depth 6cm (7L) with an identical shelter placed in the bottom-right of each arena (14 x 6.5 x 6.5 cm) were used (**Figure 10**). Tagged fish from each tank were collected from the trial tank and transferred in identical white transfer buckets with closed tops and then placed in random order into individual arena shelters through a top compartment (4cm diameter) which was then closed, and the main shelter door (a sliding opaque trapdoor) was kept shut, closing off access to the arena. All four arenas were filmed simultaneous and continuously for 25 minutes. The first 5 minutes, the fish remained shut inside the shelter for a period of acclimation, after which the door to each arena shelter was lifted simultaneously and filming continued for a further 20 minutes uninterrupted. Each individual arena was virtually divided into four zones using the EthoVision XT software. These zones were called Shelter, Entry, Border and Centre (**Figure 11**). The centre zone was considered high-risk and staying close to the border edges of a space was considered more cautious and an indicator of a shyer individual (Benhaïm et al., 2020; Dahlbom et al., 2011). Variables used to characterise OFT behaviour for the trait of boldness were as follows: Latency (s) to emerge-time taken to exit the shelter. Time spent in each zone (s) (Shelter, Entry, Border, Centre) respectively. Mean distance from shelter (cm). Number of returns to shelter. Cumulative duration spent highly mobile (s). Distance moved (cm). Mean velocity (BL s^{-1}). Absolute angular velocity (° s-1). Standard statistically techniques were followed and described in *Paper I*.

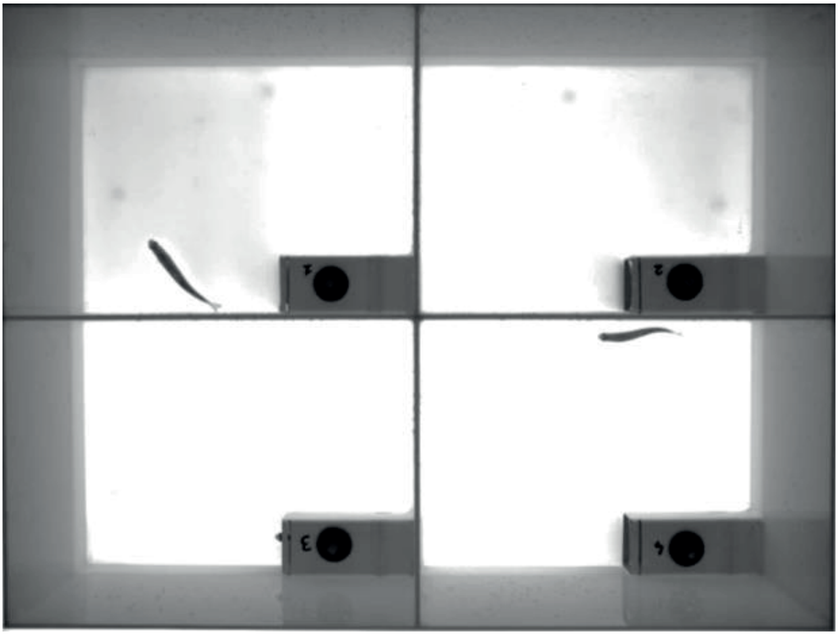

Figure 10. Open Field Test arena with shelter, used for observation of the behavioural trait of boldness.

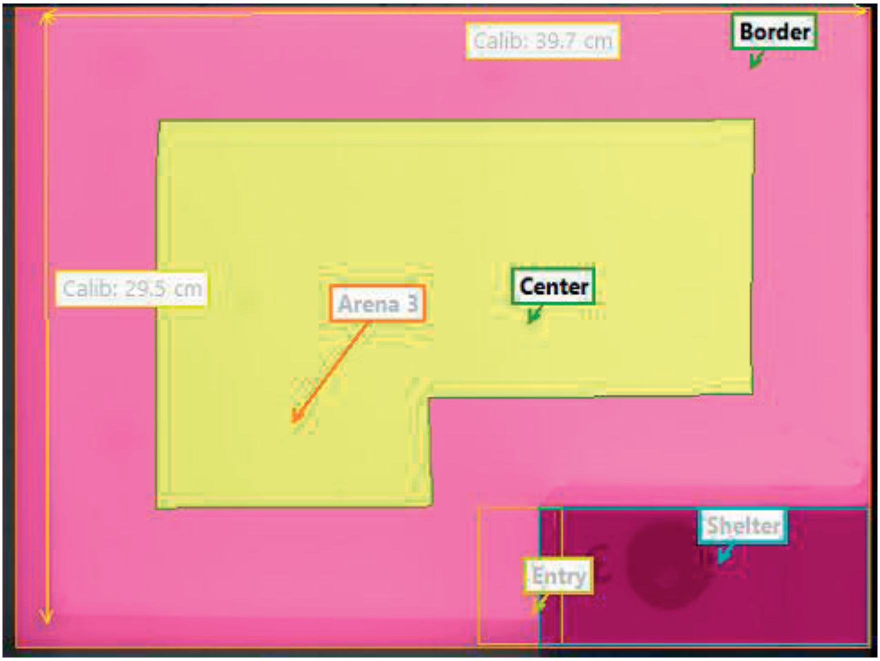

Figure 11. Four virtual zones outlined in EthoVision XT as Shelter, Entry, Border, and Centre for the OFT with shelter test.

3 Results

3.1 Paper I

This multi-discipline study examines the impact of two novel enhancements of soybean meal (SBM), an enzyme pre-treated SBM (ETS), and a SBM diet with added fructooligosaccharide (FOS) (USP) on growth performance, gut microbiome, and individual behaviour, including response to stress stimulus and behaviour traits relevant to welfare of farmed salmonids (boldness and exploration). These enhanced protein sources were compared with conventional SBM (US) and fish meal (FM) in diets fed to freshwater phase Atlantic salmon (*Salmo salar*). The inclusion of SBM was 25% in all SBM-based diets. Results showed that that FM fed fish had significantly better growth performance than US-SBM fed fish, but that no significant difference was found between fish fed FM and the two enhanced SBM-based diets, ETS and USP. The gut microbiome community was distinctly different among fish fed the different diets. Fish fed the FM diet had a significantly higher diversity and evenness of the gut microbiome community assemblage, while ETS-SBM had the lowest community diversity and evenness compared to the other diets. Examination of the community at the genus taxonomic level showed that this was due to the presence of the lactic acid bacteria (LAB), *Enterococcus*, which had a high relative abundance in ETS fed fish but did not exclusively dominate the community. Overall, there was a greater presence of LABs in all SBM fed fish compared to FM fed fish, but there was a greater presence of bacterial genera which have been associated with faster growth in salmonids in the FM diet compared to any of the SBM diets. There were no significant differences in the behavioural traits measured across the fish fed the different diets in this study, however, a consistent trend that FM fed fish were bolder, more active explorers, both characteristics associated with a proactive coping style, which suggests they may be better suited for intensive farming and therefore have better welfare. A behavioural syndrome was identified for FM, USP-SBM and ETS-SBM fish but not for US-SBM fish indicating that they may be experiencing different pressures than other fish. The repeatability of these behavioural traits was high for all diets, but was highest for FM fed fish, which may also indicate better welfare for these fish. The connection between dietary treatments and the impact to the gut-brain-axis, and consequently production and welfare warrants further study.

3.2 **Paper II**

This study examined the impact of novel processing of black soldier fly (BSF) larvae (*Hermetia illucens*) reared on food waste, on growth performance, gut health and gut microbiome of FW phase Atlantic salmon. The novel processes were dechitination (BSFC-) and fermentation with the probiotic bacteria *Pediococcus acidilactici* (BSFC+P+). These processed BSF were compared to non-processed BSF (BSFC+), soy protein concentrate (SPC), and fish meal (FM) diets. All BSF products were included in diets at 10%. Results showed that there was no significant difference in growth performance or gut health of fish fed any of the study diets. The gut microbiome community assemblage of fish was significantly affected by the different diets. The BSFC+ diet resulted in a community with the greatest diversity, richness, and evenness compared to the other diets, indicating that at this inclusion level of BSF, chitin may have prebiotic value for the gut microbiome. The fermented BSFC+P+ gave the lowest values for gut microbiome community diversity, richness, and evenness. This result viewed at the genus taxonomic level indicated that the fish fed BSFC+P+ had a gut microbiome exclusively dominated by the bacteria *Exiguobacterium,* a chitinase active bacteria that is potentially beneficial, but in such an unbalanced gut microbiome community may indicate dysbiosis, an undesirable condition for fish resilience. This same bacterium was present in high relative abundance in the BSFC- fed fish gut, without exclusion of other genus, suggesting potentially positive impacts without dysbiosis for fish fed this diet. This suggests that the impact of fermentation of insect proteins for salmon at such an early stage requires further investigation. Overall, this study suggests that at lower inclusion levels of BSF, additional processing is not necessary, on the contrary, the presence of chitin may have additional beneficial effects on the gut microbiome. This study indicates that BSF larvae for salmonid diets is a promising candidate for aquafeed inclusion.

3.3 **Paper III**

This study examined the impact of increasing inclusion levels of torula yeast (*Cyberlindnera jadinii*), cultured on lignocellulosic biomasses from hardwood side streams, as an alternative protein for salmonids. The yeast was added as a replacement of fish meal (FM) and a plant protein blend (MIX) at a yeast inclusion level of 0% (FM00 & MIX00), 10% (FM10 & MIX10) and 20% (FM20 & MIX20) of the protein from FM and MIX were fed to freshwater Atlantic salmon. The impact on growth performance and gut microbiome were investigated. Results showed that the highest yeast inclusion level FM20, could replace FM without adverse effects on growth performance, and with increased beneficial effects on gut microbiome that could have potential immune-modulating effects for the fish. However, when fed in combination with MIX proteins, the 10% yeast diet (MIX10) supported higher growth than 20% yeast (MIX20) without reducing the presence of desirable LABs. This study supports the continued investigation of torula yeast as a promising alternative protein in diets for Atlantic salmon and indicates that optimising both the combination of conventional and alternative dietary proteins and the inclusion levels of each, warrants further research attention.

3.4 Paper IV

This cross-discipline review mapped the ecosystem services (ES) provided by seaweed, perennial grasses, and legumes, which are all considered under-utilised production systems in Europe. The existing knowledge of the use of biomasses derived from these production systems in circular bioeconomy and aquafeed research was reviewed to facilitate the environmentally, and economically sustainable exploitation of seaweed, perennial grasses, and legumes to meet the resource needs of the growing aquafeed industry. The findings indicate that all these production systems provide highly valuable ES. Many of the biomasses derived from these systems are considered promising candidates for proteins or feed additives for aquaculture. However, this review also found that despite circular bioeconomy models being widely considered a promising mechanism to meet the feed sector needs and to utilise such resources, a knowledge gap existed to support the development of such supply chains. This review presents a theoretical circular bioeconomy model that could connect these under-utilised production systems to the aquafeed industry while supporting valuable ecosystem services and highlights the need for more research attention on both the biorefinery processes of such biomasses, and the application in aquafeed diets to facilitate establishment of such a model.

4 Discussion

Atlantic salmon is the 9th most farmed species globally, and intensive farming both onshore and offshore is expanding (FAO, 2020). If the industry is to provide a sustainable food source, it must reduce its environmental impact and improve the nutritional value of aquafeeds for salmonids (Naylor et al., 2021). This doctoral thesis aimed to optimise the protein supply in diets for juvenile Atlantic salmon (*Salmo salar*), both from protein sources currently available to the aquafeed sector and from key alternatives.

The combined findings of the papers contributing to this thesis are summarised in **Figure 12**, and organised according to the first, second and third priorities identified for this thesis. The main findings are that firstly, the application of SBM in juvenile salmonid diets can be improved through the addition of FOS and enzyme pre-treatments, secondly, alternative proteins from yeast and BSF at moderate inclusion levels are well suited for juvenile Atlantic salmon and do not require further processing and thirdly, environmental impact of the aquafeed sector could be mitigated through circular bioeconomy strategies that reduce waste and support the sustainability of supply. The studies in this thesis highlighted key knowledge gaps that need to be addressed to facilitate the sustainable expansion of Atlantic salmon aquaculture. The following discussion explores these key findings in context with the existing literature, evaluates the impact to the field of Atlantic salmon aquaculture, and highlights new priorities for future research attention.

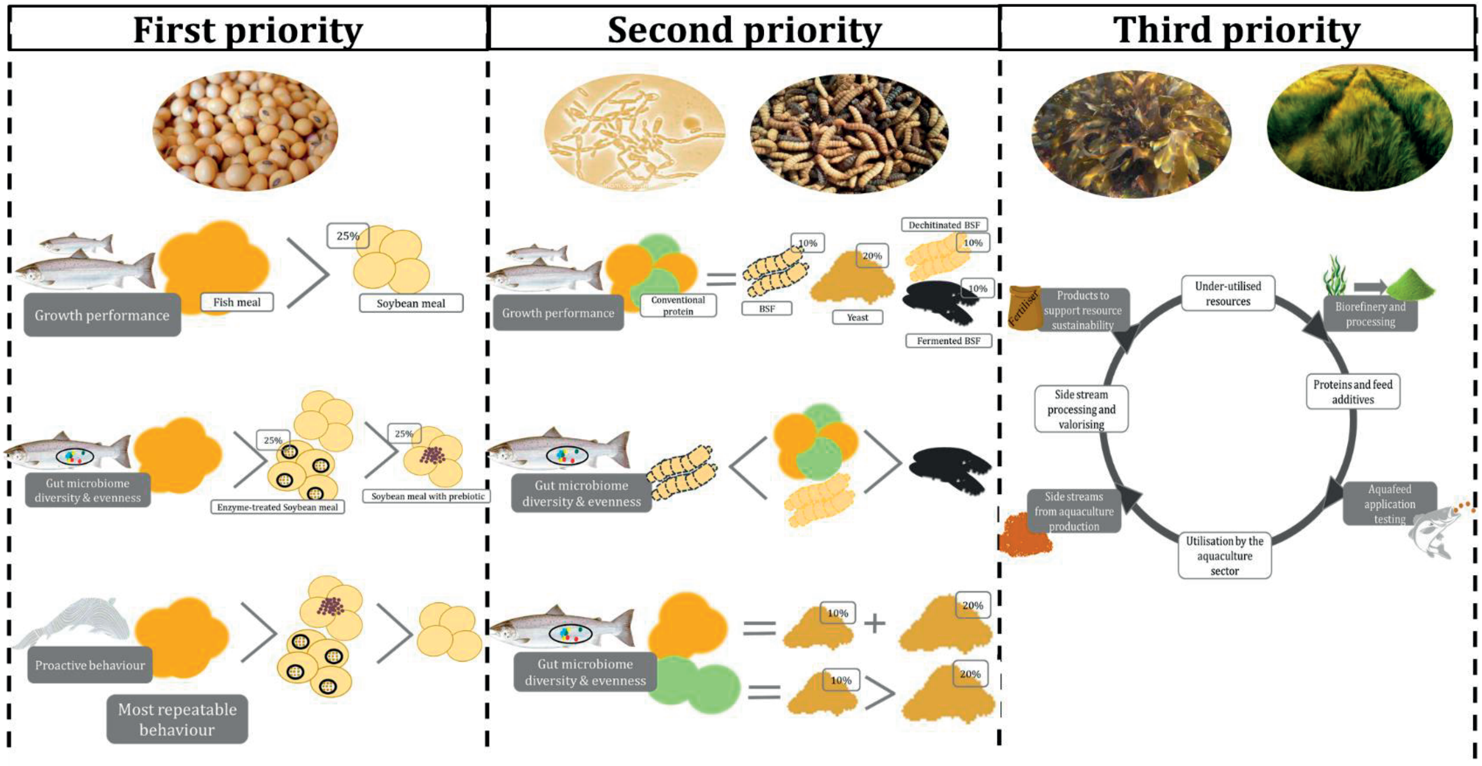

Figure 12. Summary of the key findings of all four papers within the doctoral thesis. The first priority was to characterise and optimise the proteins that are currently available to the aquafeed sector, the second priority, to characterise and optimise alternative proteins that have potential for the aquafeed sector, and the third priority, to investigate the mechanism for sustainable exploitation of new protein sources. Percentages displayed show the inclusion level of protein sources used.

4.1 Proteins and growth performance

Growth performance has traditionally been used as an indicator to determine what feed formulations and ingredients are the most suitable to optimise production in aquaculture. The studies of *Paper I, Paper II*, and *Paper III* in this doctoral thesis investigated this measure as a primary indicator of the suitability of enhancing SBM, replacing conventional proteins with alternative proteins and processing alternative proteins for Atlantic salmon aquaculture.

Processing and enhancing plant meals, especially SBM to improve growth performance of salmonids has received growing research attention. *Paper I* indicated that both addition of prebiotic FOS and enzyme pre-treatment targeting NSP in SBM, could improve growth of FW salmonids. Similar improvements were seen in SW Atlantic salmon fed diets to reduce ANFs and oligosaccharides (Refstie et al., 1998), and in Rainbow trout with FOS supplemented diets (Ortiz et al., 2013). Conversely, an existing study that fed enzyme pre-treated SBM to Rainbow trout raised in saltwater did not find any growth performance benefits (Denstadli et al., 2011). In other aquaculture species such NSP-targeted enhancements also supported growth benefits, in Japanese seabass (*Lateolabrax japonicus*) fed mixed plant meals (Ai et al., 2007), white-spotted snapper (*Lutjanus stellatus*) fed macroalgae (Zhu et al., 2016), and the supplementation of plant protein with the probiotic bacteria (*Bacillus pumilus*) for Nile tilapia (*Oreochromis niloticus*) (Hassaan et al., 2021). Such enhancements are less effective at improving growth when applied to SPC (Denstadli et al., 2007), and FM (Grisdale-Helland et al., 2008) suggesting these enhancements are best suited to unprocessed plant ingredients. Other enhancements of SBM diets evaluated in the existing literature include fermentation of SBM for Rainbow trout which supported growth performance benefits (Yamamoto et al., 2010). Such enhancements to SBM in parallel with development of broodstock programs that select for SBM-tolerant salmonid genetic lines offer a promising strategy to optimise growth benefits (Abernathy et al., 2017) and warrant further attention.

Alternative proteins from BSF and yeast as partial replacements for conventional proteins in salmonid feeds, have been evaluated for their impact on growth performance of salmonids. *Paper II* and *Paper III* indicated that BSF at 10% inclusion as a replacement for FM in mixed protein source diet, and up to 20% of the yeast, *C. jadinii* could replace FM in a FM-based diet and plant meals in a mixed protein source diet did not have any adverse effects to growth performance. Interestingly, 10% yeast in a mixed protein source diet supported better growth than 20%. Existing studies have observed that even at high inclusion levels BSF supported comparable growth performance to conventional proteins at 30% inclusion (Fisher et al., 2020), and 85% protein replacement (Belghit et al., 2018) respectively, in FW Atlantic salmon. In SW Atlantic salmon up to 100% FM replacement with BSF was possible without adverse effects to growth (Belghit et al., 2019; Lock et al., 2016). Yet, others have shown best growth results at moderate inclusion levels (Henry et al., 2015; Weththasinghe et al., 2021b). It is possible that differences in nutritional composition of BSF, which may be influenced by the growth substrate (Chia et al., 2020), or the pre-processing method used (Weththasinghe et al., 2021a) may influence optimal inclusion but this requires further investigation. It will be equally important to standardise the nutritional composition and to determine the optimal processing of BSF to maximise inclusion, and this may be different for FW and SW phases. The findings for yeast are widely in agreement with the existing literature, that *C. jadinii* could replace up to 40% of FM proteins without compromising growth for FW Atlantic salmon (Øverland et al., 2013). *Paper III* is not the first study to indicate that moderate inclusion levels may be preferable to higher inclusion levels of torula yeast to support optimal growth performance, the same trend was seen in Tilapia (*Oreochromis mossambicus*) (Olvera-Novoa et al., 2002). It has been suggested that higher inclusion levels of torula yeast are most compatible in combination with marine protein (Øvrum Hansen et al., 2019). The inclusion level of alternative proteins and which conventional proteins they are most compatible with are important considerations to optimise growth performance. Understanding why moderate inclusions of alternative proteins in mixed formulation diets perform better than higher inclusion levels will be valuable for future feed development.

Attempts in *Paper II* to optimise BSF for FW salmon diets by dechitination and fermentation with the probiotic bacteria, *Pediococcus acidilactici*, while comparable with conventional proteins did not improve growth performance compared to unprocessed BSF. This suggests that chitin at least at 10% inclusion level of BSF is not limiting to growth as had been proposed by existing research (Kroeckel et al., 2012; Olsen et al., 2006; Weththasinghe et al., 2021a; Xiao et al., 2018). Fermentation of insects in the existing literature has improved growth performance of several carp species (*Catla catla, Cirrhinus mrigala, Labeo rohita, Hypophthalmychthys molitrix*) (Rangacharyulu et al., 2003), and in broiler chickens fed only fermented silkworm pupae silage (Rao, R et al., 2011) and fermented sago larvae as a replacement for FM (Sjofjan and Adli, 2021). Since *Paper II* is the first evaluation of these processing methods for BSF and for salmonid diets, this indicates, at least at 10% inclusion level and in the FW phase, that there is no growth benefit of such additional processing. However, it will be important to evaluate such methods both at higher inclusion level and in SW Atlantic salmon as results may differ and growth benefits might be observable.

Cumulatively, these findings address the research objective, and show that there are growth performance benefits associated with using some, but not all, of the diets and dietary treatments studied. Since processing of ingredients increases costs for the aquafeed sector, it is vital that the benefits of processing have sufficient value (Denstadli et al., 2011; Galkanda-Arachchige and Davis, 2020). The studies in this thesis indicate that enhancing SBM may add value for FW Atlantic salmon diets, but processing BSF proteins at least at 10% inclusion, may not be necessary, making it very cost-efficient (Weththasinghe et al., 2021a). However, these studies also highlight that growth performance, while a valuable measure for the aquaculture industry, taken alone is not enough to determine the suitability of a protein type, processing method or inclusion level for aquaculture feeds.

4.2 Proteins and gut microbiome

All the research studies (*Paper I*, *Paper II*, and *Paper III*) in this doctoral thesis combined growth performance measures with a characterisation of the gut microbiome community assemblage. There is substantial evidence that microbiota in the GI tract have a key role in the nutrition that fish can gain from feed ingredients (Ghanbari et al., 2015; Llewellyn et al., 2014; Merrifield and Rodiles, 2015), and that particularly in the digesta, the microbiota present is highly influenced by feed ingredients (Gajardo et al., 2017; Li et al., 2021) and early developmental stages for salmonids are especially sensitive to protein alteration (Michl et al., 2017, 2019). Characterising the gut microbiome of fish fed different proteins type and processing type is also important since there may be indirect consequences for the immune system of fish which can impact production and resilience (Gajardo et al., 2016; López Nadal et al., 2020; Yukgehnaish et al., 2020).

The papers of this thesis concur with the literature and demonstrate the same trend for the first time in juvenile FW Atlantic salmon that both different protein types and different protein treatments result in distinctly different gut microbiome communities. Differences have been found in salmonid gut communities between marine protein diets and plant protein diets (Gajardo et al., 2017; Michl et al., 2019, 2017), between conventional proteins and alternative proteins from insects (Bruni et al., 2018; Huyben et al., 2019), and yeast (Huyben et al., 2017). Existing research has also observed difference driven by enhancements and processing methods, such as the addition of prebiotic FOS in sea bream (*Diplodus sargus*) feeds (Guerreiro et al., 2018), the addition of chitin in Atlantic salmon (Askarian et al., 2012), the enzyme pre-treatment of plant meals in combination with the probiotic bacteria (*B. pumilus*) in Nile tilapia (*O. niloticus*) (Hassaan et al., 2021) and fermentation of SBM for Atlantic salmon (Catalán et al., 2018). In gut microbiome research, communities with high diversity and evenness are considered to have more immune resilience and have the potential to support more functional diversity for the fish host (Apper et al., 2016; Infante-Villamil et al., 2021). In the studies herein, in *Paper I*, FM diets supported higher gut microbiome diversity and evenness than any SBM diet, in *Paper II,* non-processed BSF had higher diversity and evenness than conventional proteins (FM and SPC) and compared with either processed type of BSF, and in *Paper III*, diversity and evenness were comparable between FM and FM with yeast up to 20%, but in a mixed protein source diet, addition of yeast led to lower diversity. In existing research there are different findings, for example Gajardo et al, (2017) showed that feeding SBM and wheat gluten diet to SW Atlantic salmon gave greater gut microbiome community diversity than those fed FM and in juvenile brown trout (*Salmo trutta*) plant diets also supported greater gut microbiome diversity than FM diets (Michl et al., 2019). In another study SPC also increased the gut microbiome diversity compared to FM in SW phase Atlantic salmon (Green et al., 2013). However other studies concur with the findings of this thesis that FM does support greater diversity and community evenness than SBM in salmonids (Bruce et al., 2018; Reveco et al., 2014). Existing studies for BSF have also shown higher diversity compared to conventional proteins (Li et al., 2021), and that yeast at moderate inclusion (<20%) supports similar gut microbiome community indices to marine protein sources, for example in the gilthead sea bream (*S. aurata*) (Rimoldi et al., 2020) and in Rainbow trout (*O. mykiss*) (Huyben et al., 2017). In *Paper I* and *Paper II,* certain processing methods, enzyme pre-treatment of SBM and fermentation of BSF both influenced a notably low diversity and community evenness in the juvenile Atlantic salmon gut which required investigation at a high taxonomic resolution.

Fermentation of BSF led to the exclusive establishment of the chitinase-active bacterium (*Exiguobacterium*) and for enzyme pre-treated SBM there was a high (but not exclusive) abundance of the LAB, *Enterococcus*. Both *Exiguobacterium* and *Enterococcus* are microbiota that could have positive functional benefits in the fish gut. *Exiguobacterium* helps to degrade chitin and is found in wild fish allowing them to access nutrition from insect sources (Salas-Leiva et al., 2017; Webster et al., 2020). Chitinase activity can have immune benefits for fish (Zhang et al., 2012) and has been linked to improved growth performance in goldfish (*Carassius auratus*), and in some species even inhibition of pathogen growth (Jinendiran et al., 2019). Further, *Enterococcus* has been associated with growth and immune benefits in fish (Alshammari et al., 2019). This raises the question, is lower diversity and evenness, when driven by the presence of bacteria associated with positive benefits for fish hosts, a desirable or undesirable state for the gut microbiome of farmed fish? In the case of fermented BSF, the community is exclusively dominated by this single microbiota and indicates the presence of undesirable dysbiosis (Infante-Villamil et al., 2021) however, while unbalanced, this case was not so extreme as the enzyme pre-treated SBM, bringing both potential benefits and reduced risk of dysbiosis. However, these findings do indicate that such gut-targeted processing should be applied with caution at such early developmental stages in salmon and monitoring for dysbiosis is recommended. Furthermore, in existing research on fish gut microbiome, LABs have been associated with benefits to the fish host (Merrifield and Carnevali, 2014; Ringø et al., 2020). Consistently in the studies herein, all LABs detected across *Paper I*, *Paper II*, and *Paper III*, *Lactobacillus*, *Enterococcus*, *Leuconostoc* and *Weissella*, were all in higher relative abundance in fish fed diets containing soy derived proteins (SBM and SPC) compared with fish fed FM, BSF or yeast diets. This finding is consistent with much of the existing literature for the impact of soy derived proteins on fish gut microbiome where enrichment of LAB groups has also be detected (Gajardo et al., 2017; Michl et al., 2019). Thus, these is a need to determine the threshold for a trade-off between the adverse impacts of soy derived proteins and the benefits of LABs to inform optimal feed formulations.

Cumulatively, the findings of *Paper I, Paper II* and *Paper III* address the thesis objective, and show that benefits to gut microbiome can be gained through some, but not all, of the diets and dietary combinations studied. The studies in this thesis suggest that gut-targeted enhancements of SBM bring benefits to the gut microbiome, and that alternative proteins at moderate inclusion may bring gut microbiome benefits, especially for BSF which has prebiotic potential when chitin is left intact. These studies also highlight the need for caution when applying gut-targeted feed processing at very early developmental stages when the gut microbiome is in initial establishment stages.

4.3 Proteins and behaviour

Paper I, sought to characterise the behaviour of fish fed different enhancements of SBM compared with FM to complement the characterisation of gut microbiome and growth for these protein sources and to improve the existing knowledge on the welfare impact of conventional diets. This study demonstrated for the first time that behaviour of farmed Atlantic salmon can be influenced by the protein type and protein enhancement in feed. Results indicated that FM fed fish may be experiencing better welfare than SBM fed fish, but that gut-targeted enhancements of SBM might offer some amelioration.

Paper I of this thesis identified a trend that Juvenile Atlantic salmon fed FM diets tended to have a more proactive coping style (displaying bolder and more exploratory behaviour (Castanheira et al., 2015). While this field is in its infancy, the existing research is in agreement with this finding, that plant ingredients lead to more reactive styles than marine ingredients, in Rainbow trout (Sadoul et al., 2016) and in Senegalese sole (Ibarra-Zatarain et al., 2015), Other studies have indicated, that for fish fed diets enriched in omega-3 fatty acids for larval yellow tail (*S. quinqueradiata*) and gilthead sea bream (*S. aurata*) have stronger shoaling behaviour which for shoaling fish is likely to reduce stress (Benítez-Santana et al., 2012; Castanheira et al., 2015; Ishizaki et al., 2001). This could have important implications for the selecting ingredients and formulating diets, and there is a need to understand the nutritional profiles that might support compatible coping styles. The impact of alternative proteins on salmonid behaviour will be important to characterise to determine how it might impact coping style and consequently, welfare in intensive aquaculture systems (Huntingford and Adams, 2005; Ibarra-Zatarain et al., 2015), and would be important to enhance studies such as *Paper II* and *Paper III*.

The existing research has indicated that proactive fish may be more suited to the environmental conditions in intensive aquaculture (Huntingford and Adams, 2005), and where there would be productivity gains for fish that feed more readily after moving tank (Øverli et al., 2007), are more tolerant to stress (Höglund et al., 2008), and more efficient feeders (Martins et al., 2006). It has been suggested too, that when feed is not a limiting resource, proactive fish will grow better than reactive individuals (Martins et al., 2005), as was indicated by *Paper I*. Additionally, *Paper I* results indicate that FM fed fish were not only more proactive, but they also had the most repeatable behaviours, suggesting they are more able to consistently express their natural behaviour, an measure of good welfare under the five freedoms definition (Webster, 2001). Monitoring behaviour is becoming more common place in salmon aquaculture for both welfare monitoring and feed management (Føre et al., 2011; Macaulay et al., 2021) and as video and machine learning tools become more sophisticated, it may be possible to monitor coping style at the individual and group level for early indicators of fish stressors, which will in turn, support more resilient fish and better farming practices (Colditz and Hine, 2016). This would require further characterisation of coping style in salmon and determination of optimal behaviour for farmed fish productivity and welfare. Furthermore, since coping style is strongly linked to hormonal and endocrine responses, such measures could provide further insight into the welfare status of farmed salmon, in different farm and feeding conditions (Castanheira et al., 2015; Koolhaas et al., 1999; Magnhagen, 2012). In *Paper I*, the only fish that did not display a behavioural syndrome (a correlation between behavioural traits (Conrad et al., 2011)) were those fed untreated SBM, this may conversely mean that they are less able to express natural behaviour (Webster, 2001) and that they have had to adapt to adverse conditions by becoming more behaviourally plastic during this early life stage (Hope et al., 2020; Sih et al., 2004). Since the two gut-targeted enhancements of SBM ameliorated this impact, this provides useful insight into the connection between the potential to use dietary manipulations of the gut microbiome to alter the behaviour and improve welfare. There is a growing body of research investigating how dietary changes can affect behaviour through the bi-directional signalling in the gut-brain-axis, evidence emerging in domesticated (Kraimi et al., 2019) and wild animals (Cairo et al., 2021), suggests this is an important area for future research attention that will provide valuable information for aquaculture.

Coping style may be a powerful measure to optimise selective breeding programs for Atlantic salmon, since it is also influenced by genetics (Ferrari et al., 2016). In wild fish populations, both proactive and reactive strategies exist, and each have ecological trade-offs (Skov et al., 2019), however, it is likely in already domesticated fish like tilapia and salmon that there has already been selection pressure toward fish that are more competitive, more stress tolerant and overall, more proactive individuals (Huntingford and Adams, 2005). It has even been suggested that farmed Atlantic salmon should be considered a different species from their wild counter parts, *Salmo domesticus* (Gross, 1998). This could have important consequences for welfare and productivity in commercial farms, but also is an added consideration for the environmental impact of farm escapees on wild populations, a significant problem for sea cage farming (Islam et al., 2020). It will be important to characterise the impact of domestication on salmonid coping styles and develop farms designed to suit the welfare needs of intensively farmed domestic fish, while still ensuring economically viable production.

Paper I successfully addressed the objective of this thesis and indicated that benefits to Atlantic salmon behaviour could be gained by gut-targeted enhancements of SBM compared with untreated SBM but highlighted potential differences in behaviour and consequently welfare between feeding marine protein and plant proteins. The results of this study also raised a whole host of new questions regarding the connection between coping style and gut microbiome, and how feed ingredients may influence the gut-brain axis in Atlantic salmon.

4.4 **Supply of proteins for aquaculture**

Ensuring a sustainable and resilient supply of feed ingredients will be essential to support continued expansion of Atlantic salmon aquaculture (Cottrell et al., 2020; Naylor et al., 2021). *Paper IV* investigated the potential to exploit biomasses from currently under-utilised blue green production systems (from seaweeds, perennial grasses, and legumes) to meet the protein demand for the aquafeed industry. This study highlighted that circular bioeconomies offer a promising mechanism for the aquaculture sector to access such novel proteins. *Paper II,* and *Paper III,* further demonstrated opportunities for the circular economy to supply alternative proteins to the aquaculture sector. Such models could provide a positive feedback loop to ensure the environmental and economic sustainability of supply, a factor that will be important for future-proofing supply chains against the impacts of climate change (Kardung et al., 2021; Yarnold et al., 2019).

Proteins derived from soybeans are currently the most scaled and available protein source for aquafeeds (El-Shemy, 2011). Yet there are increasingly alternative proteins that are suitable for aquafeed inclusion that require increased production scales and supply chain establishment to ensure they can be competitive options to soybeans. There are also biomasses that are not currently exploited in Europe that could provide important sources of protein and feed additives for aquaculture, such as those highlighted in *Paper IV*. Exploiting alternative proteins close to market and more novel proteins will be an important measure to reduce the European protein deficient (Smárason et al., 2019), and limit salmonid aquaculture reliance on less sustainable, or imported protein sources.

In *Paper II*, BSF larvae were reared primarily on organic food waste from potatoes, and in *Paper III*, torula yeast was cultivated on hexose and pentose sugars from lignocellulosic biomasses sourced from forestry side streams. In the existing literature, insects have also been successfully reared on a wide range of organic wastes (Bosch et al., 2019), this is of particular interest for further research, since the nutritional composition of the substrate influences the composition of the insect, which will have consequences for aquafeed inclusions (Tschirner and Simon, 2015). Yeast has also been successfully cultivated on many substrates (Sharma et al., 2018; Solberg et al., 2021), and bacterial meals which are promising sources for salmonid feed proteins, have been cultured on natural gas (Storebakken et al., 2004). *Paper IV* highlighted three sources of currently under-utilised biomasses that could support future aquaculture demand for protein and feed additives from seaweeds, perennial grasses, and perennial legumes. They could provide low-cost, high yield feed stocks with favourable amino acid profiles, and potentially valuable metabolites and compounds for animal feeds (Angell et al., 2016; Larsen et al., 2019; Morais et al., 2020; Scordia and Cosentino, 2019). Aquaculture research has evaluated only a few of the potential biomasses available, for example the seaweeds; *Porphyra purpurea* for thick-lipped grey mullet (*Chelon labrosus*)(Davies et al., 1997), and *Laminaria sp*. In the diet of Atlantic salmon (Kamunde et al., 2019), in the perennial grass, Kiku grass (*Pennisetum clandestinum*) in the diet of redbreast tilapia (*Coptodon rendalli*) (Hlophe et al., 2011) and the perennial legume alfalfa in the diet of tilapia (*Oreochromis mossambicus*) (Olvera-Novoa et al., 1990), all with promising results. *Paper IV* identified several other biomasses that require evaluation for aquafeed inclusion. There are limitations and bottlenecks that will need to be addressed for these under-utilised biomasses to be directly exploited by the aquaculture sector, research must optimise biorefinery processing and create methods for protein and compound extraction.

To facilitate a smooth transition to integrating alternative and novel proteins, research must find innovative ways to integrate existing and new value chains to optimise access to ingredients for aquaculture (Kusumowardani and Tjahjono, 2020; Little et al., 2016) in a way that concurrently meets the needs of the environment and economy. *Paper IV* highlighted the increasing political impetus for growth in circular bioeconomies (2008/56/EC European Marine Strategy Framework Directive, European Blue Growth Strategy) (Carus and Dammer, 2018). *Paper IV* presented a conceptual how a circular bioeconomic model that could be used both to directly exploit novel biomasses, but since there are substantial knowledge gaps, the paper also indicated how more rapid integration and smoother transition might be possible through indirect exploitation as illustrated in **Figure 13**. It monopolies on the opportunity to cultivate alternative protein sources on diverse substrates, in this example, insects like BSF can be reared on grasses and seaweeds (Parra Paz et al., 2015; Swinscoe et al., 2019), these biomasses could be a valuable and nutritionally consistent substrate source, which would simultaneously solve an existing limitation for insect cultivation simultaneously (Bosch et al., 2019; van Huis, 2020). Since perennial grasses are also rich in lignocellulosic biomasses (Muylle et al., 2015), there is also an opportunity for similar indirect models to support yeast production (Øverland and Skrede, 2017). Lastly, such a model supports circular opportunities to create a positive feedback loop so aquaculture wastes could boost plant biomass productivity and resilience, while simultaneously treating organic waste, and increase the value of ecosystem services provided by blue green production systems.

Paper IV addressed the research objective to review the research on under-utilised blue green productions systems in Europe and the status, and research needs to facilitate their sustainable exploitation by the aquaculture sector. *Paper II* and *Paper III* demonstrated how such circular bioeconomies might directly supply alternative aquafeed proteins. Cumulatively, this research highlighted the importance of cross-sector collaboration to ensure the success of future-resilient circular bioeconomies for aquaculture.

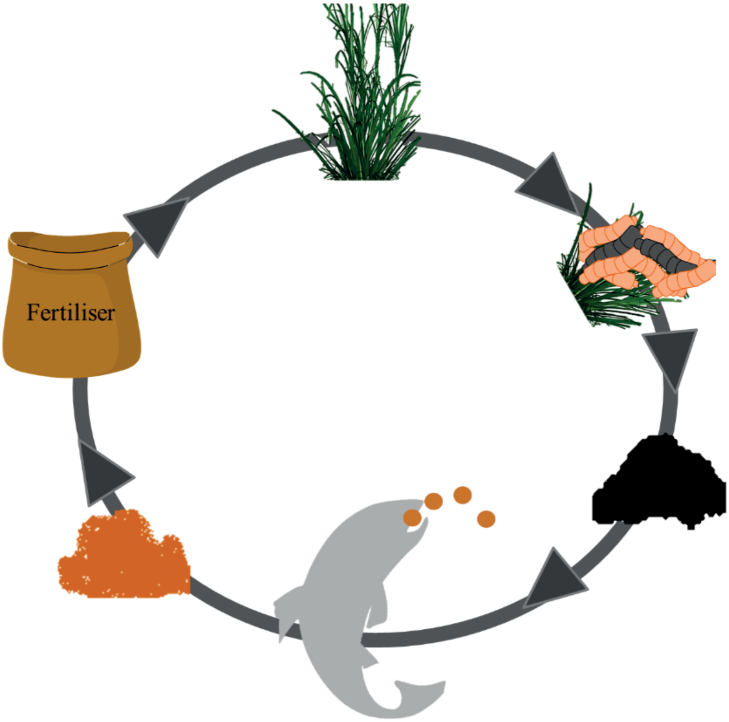

Figure 13. Conceptual circular bioeconomy model to indirectly exploit under-utilised biomasses and integrate them into the aquafeed supply chain more rapidly.

4.5 Future perspectives

The collective findings of this thesis highlight new research priorities that require future attention to support sustainable growth in Atlantic salmon aquaculture. For effective communication, these new research priorities have been divided into three key focus categories, firstly, biology and physiology, secondly, aquaculture operations, and thirdly, circular bioeconomy (**Table 3**).

Table 3. New research priorities from three key focus categories.

Biology and physiology
• Investigate gut-targeted enhancements of soybean meal, and processed BSF in SW phase salmon and at higher inclusion levels. • Investigate processing methods of yeast that may increase inclusion in mixed protein diets. • Evaluate proteins and feed additives derived from under-utilised biomasses in aquafeeds. • Identify indicator microbiota in Atlantic salmon GI tract and microbiota with key functional roles relevant to Atlantic salmon production. • Investigate how coping style and gut-brain-axis may be altered by different processing methods of soybeans and by different protein sources.
Aquaculture operations
• Develop welfare indicators based on coping style and monitoring technologies. • Investigate how the gut-brain-axis of Atlantic salmon might be affected by domestication, and what the consequences for welfare. • Develop salmonid selection programs based on coping style. • Further develop selection programs for SBM tolerant salmonids.
Circular bioeconomy
• Identify organic side streams in Europe that could be exploited for aquafeeds. • Commence dialogue between researchers and policy makers to safely utilise new organic substrates for production of alternative proteins. • Create clear guidelines for how to establish circular bioeconomies for aquaculture.

5 Conclusions

The global aim of this thesis was to optimise the protein supply in diets for juvenile Atlantic salmon, from key protein sources currently available on the market and promising alternatives that could meet the growing demands of the aquaculture sector without further adverse environmental impacts. Through a multi-discipline approach, three research papers and one review successfully addressed this aim and contributed key knowledge to the field of sustainable aquaculture:

- Gut-targeted enhancements of soybean meal have potential to improve use in Atlantic salmon aquaculture.
- Proteins from non-marine sources may influence behavioural traits and coping styles that have relevant consequences for farmed salmon welfare.
- Changes in protein source and any enhancements or processing of protein source have significant consequences for the microbiome community assemblage of juvenile Atlantic salmon, and such alteration may have consequences for immune resilience.
- Soybean meal and soy protein concentrate increase the presence of lactic acid bacteria in the GI tract of FW Atlantic salmon.
- Black soldier fly larvae and torula yeast are promising candidates to replace fish meal in FW salmon feeds.
- The optimal inclusion of torula yeast in FW Atlantic salmon diets is 10% when it replaces plant proteins.
- The chitin present in black soldier fly larvae at moderate inclusion levels is not a limiting factor and may have prebiotic value for juvenile Atlantic salmon.
- Circular bioeconomies provide direct and indirect opportunities to integrate under-utilised blue green biomasses and alternative proteins into aquafeed value chains and can create positive feedback for environmental resilience and sustainable exploitation.

Finally, this thesis highlighted key focus categories across these multiple disciplines and new research priorities to support sustainable Atlantic salmon production (**Section 4.5**).

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Paper I

Enhancement of soybean meal alters gut microbiome and influences behaviour of farmed Atlantic salmon (*Salmo salar*)

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Abstract

Atlantic salmon (*Salmo salar*) is one of the most domesticated, and selected, intensively farmed finfish globally. The increasing scale of production requires high quality and suitable protein for the formulated feeds of this carnivorous fish. The aquaculture sector has become increasingly reliant on soybean meal (SBM) and soy-derived proteins, and they have become the dominant protein in commercial aquafeeds due to their low-cost, availability and favourable amino acid profile. For Atlantic salmon, the inclusion of soybean meal, and soy protein concentrate (SPC) in certain combinations has adverse impacts on gut health, which has consequences for health and welfare of these farmed fish and limits the use

of soy products in salmonid diets. This study sought to address this problem by evaluating two gut health-targeted enhancements of SBM for inclusion in freshwater phase salmon diets, enzyme pre-treatment (ETS) and addition of fructose oligosaccharide (USP), respectively. This study took a multi-discipline approach, investigating both the impact to growth performance, gut microbiome, and behaviours relevant to welfare in aquaculture. The findings of this study suggest that both enhancements of SBM provide benefits for growth performance compared with conventional SBM, alter the gut microbiome community and in the case of ETS increase the presence of the lactic acid bacteria *Enterococcus*, and for the first time the impact of marine proteins and plant proteins on the coping style of salmon was indicated. Fish fed SBM showed a tendency for more reactive behaviour compared to those fed a marine protein control. All fish had a similarly low response to elicited stress. SBM fed fish also had lower repeatability of behaviour and US fed fish were the only group that did not display a behavioural syndrome, which may have implications for welfare of intensively farmed fish which is discussed.

1 Introduction

In 21st century, Atlantic salmon (*Salmo salar*) has become one of the most intensively farmed finfish in the world and the industry continues to expand as consumer demand and global population grow (FAO, 2020; Godfray et al., 2010). Unlike many intensively farmed fin-fish, Atlantic salmon is a carnivore and has a high dietary protein demand (NRC, 2011). Sourcing protein for inclusion in salmon feeds has become a major challenge for the aquaculture sector (Naylor et al., 2021). Historically, fish meal dominated the aquafeed market, but increased pressure on wild-capture fisheries and increased costs have resulted in a shift in the fish feed towards use of more plant based ingredients such as soy protein sources (Naylor et al., 2009; Tacon and Metian, 2015). Soybean meal (SBM) gained initial popularity because it is highly available, cheap and has a favourable amino acid profile for farmed fish (Gaitlin III et al., 2007). However, for Atlantic salmon, soybean meal can have adverse consequences to gut health, and triggers soybean meal induced enteritis (SBMIE) attributed to the presence of a wide range of anti-nutritional factors (ANFs) such as saponins and non-starch polysaccharides (NSP) (Ao and Choct, 2013; Baeverfjord and Krogdahl, 1996; Hedrera et al., 2013; Hu et al., 2016; Knudsen et al., 2008). These ANFs adversely alter the distal gut morphology of salmon (Å. Krogdahl et al., 2003; Romarheim et al., 2011a), reduce nutrient digestion and absorption (Booman et al., 2018), adversely alter the gut microbiome community (Kononova et al., 2019) and consequently compromises the immune resilience and welfare of fish (Sadoul et al., 2016; Talwar et al., 2018). To address this problem, the salmon industry now primarily uses soy protein concentrate (SPC) an alcohol extracted soy product that reduces the ANFs and this the inflammatory effect of SBM, but has still been observed to adversely affect the gut health and gut microbiome of Atlantic salmon (Desai et al., 2012; Green et al., 2013a; Metochis et al., 2016). Applications of such treatments increase the cost for the aquafeed sector, thus there is a need to ensure there is a tangible benefit for production or welfare (Denstadli et al., 2011).

The gut microbiome of fish has been linked to key traditional measures of aquaculture productivity such as growth (Perry et al., 2020), immune development and disease resistance (Gajardo et al., 2016; López Nadal et al., 2020; Yukgehnaish et al., 2020), metabolism (Dvergedal et al., 2020), and digestion and nutrients available to the fish (Ghanbari et al., 2015; Llewellyn et al., 2014). The composition of the gut microbiome is in turn influenced by the host´s environment (Dehler et al., 2017a), genetics (Smith et al., 2015), and feed (Gajardo et al., 2017). In juvenile salmonids, the gut microbiome is particularly malleable to alterations in dietary protein source (Michl et al., 2019, 2017a) and this can influence the establishment and development of the gut microbiome into the adult developmental stage (Dehler et al., 2017b). When salmonids are fed diets containing SBM or SPC, the composition of the gut microbiome is distinct from fish fed marine diets, bacteria associated with inflammation can increase, increase the bacterial diversity and an imbalance in the community which has been linked to poor intestinal health (Desai et al., 2012; Green et al., 2013a; Reveco et al., 2014). Increasingly, aquaculture research is highlighting the potential of dietary treatments that can have functional benefits to the host by altering the gut microbiome. Such treatments include the application of probiotics, prebiotics, synbiotics, fermentation and enzyme-treatment of ingredients (Drew et al., 2007; Kirk et al., 2002; Kiron, 2012; C. Li et al., 2020; Ringø et al., 2020). Prebiotics are non-digestible fibres that are not directly used by the host, but act as a nutritional source to support the proliferation of desirable gut microbiota (Ganguly et al., 2013), they are widely used in human foods, terrestrial farming and have high acceptance for application to aquaculture diets (Ringø et al., 2010). In aquaculture, fructose oligosaccharides (FOS), and mannan oligosaccharides (MOS) have been used in feeds to provide gut microbiome-enhancing substrates and even growth performance (Akrami et al., 2013; Grisdale-Helland et al., 2008). Enzyme treatment has also become a popular processing method to improve the value of proteins for animal and aquaculture feeds (Kirk et al., 2002). Enzyme treatment of plant proteins such as SBM has been used to breakdown long-chain carbohydrates such as NSPs to improve their nutritional value for dietary inclusion (Jacobsen et al., 2018; S. Li et al., 2020). This has a potential added benefit, as these breakdown products include, shorter chain oligosaccharides, which in fish diets are not utilised by the host, but may have a prebiotic value to the gut microbiome of the host (Bedford, 2019).

There is a growing understanding of the role of gut microbiome not just in traditional measures of productivity for aquaculture but also the observation of the bi-directional link between the gut microbiome and the central nervous system of animals including fish, which has been dubbed, the gut-brain-axis (Foster et al., 2017; Martin and Mayer, 2017). In fish there is evidence that the gut microbiome influences swimming behaviour (Borrelli et al., 2016), feeding behaviour (Perry et al., 2020) and social behaviour (Soares et al., 2019) and behaviour is an early indicator of changes in environment (Martins, Catarina, I et al., 2012). Thus, monitoring behaviour is a valuable tool for managing welfare (Sneddon, 2007) a growing concern for the aquaculture sector (Huntingford and Kadri, 2014; Solgaard and Yang, 2011).

In fish, five behavioural axes have been identified which are relevant for welfare in aquaculture, boldness, exploration, sociability, activity, and aggression (Castanheira et al., 2017; Conrad et al., 2011). When an individual displays consistent characteristics across different situations and times, this is called their coping style of personality (Koolhaas et al., 1999). Boldness is a measure of predisposition to take risks (Benhaïm et al., 2020) and individuals can be categorised on a continuum from bold to shy, exploration is a measure of predisposition to engage with a novel environment or object (Martins et al., 2012) and individuals can be categorised on a continuum from active explorers to cautious explorers (Sih et al., 2004). An individual that is bolder, or more active explorer is considered to have a coping style that is proactive and a shyer, or a cautious explorer is considered to have a coping style that is reactive (Koolhaas et al., 1999; Øverli et al., 2006). If there is a correlation between different behaviour traits at the individual level, this is known as the behavioural syndrome which can be associated with welfare and freedom to express natural behaviour (Conrad et al., 2011; Huntingford et al., 2006; Webster, 2001). Behavioural traits can be altered by dietary ingredients making them important indicators to monitor (Borrelli et al., 2016; Ishizaki et al., 2001; Sadoul et al., 2016). In juvenile Senegalese sole (*Solea senegalensis*), replacing fish oil with vegetable oils resulted in significantly more reactive individuals (Ibarra-Zatarain et al., 2015). It will be important to understand how dietary treatments affect the coping style and stress response of juvenile Atlantic salmon since it could have impacts on the productivity, fitness and welfare of farmed fish (Castanheira et al., 2015).

The objective of this study was to optimise the application of SBM in farmed Atlantic salmon diets and to elucidate the impact of novel enhancements of SBM (enzyme pre-treatment and addition of prebiotic) on growth performance, gut microbiome, and behaviour. This study hypothesises that these novel enhancements will benefit salmonid aquaculture by supporting high growth performance, increasing the presence of desirable bacteria in the gastro-intestinal tract and improve fish welfare compared with conventional SBM. This study addresses important knowledge gaps for both the aquaculture industry and the field of applied microbiology in a commercially relevant model organism.

2 Materials and Methods

2.1 Experimental animals and study design

Atlantic salmon (*Salmo salar*) hatched by Stofnfiskur Ltd. (Vogar, Iceland) were incubated at 5.5°C and eyed eggs were transferred to Laxar ehf. (Kópavogur, Iceland). Fish were raised to first feeding using standard commercial techniques and start-feed diet BioMar Inicio-plus (United Kingdom) of 0.5mm pellet size and 12°C water temperature. Fry were transferred to Verid aquaculture station of Hólar University (Saudárkrókur, Iceland) where they were acclimated for 6 weeks prior to the start of the experiment. All fish within the experiment were individually weighed and measured under anaesthetic (2-phenyoxyethanol of 300ppm) following a 24-hour fasting period. Fish were split into 12 identical 20L-White cylindrical PVC tanks, in triplicate for each feed treatment. Each tank contained 40 individual fish with initial weight of 2.2±0.4g. Fish were maintained at 11±1.9°C under continuous light of 250±50 lux and 90±10% oxygen saturation. Fish were fed with the experimental feed treatments for 70 days. The experiment was performed following Icelandic guidelines and within the permits and licenses of Verid aquaculture station.

2.2 Experimental feeds and feeding

There were four feed treatments formulated for this study. A fish meal-based control (FM) diet, an untreated soybean meal (US) diet, an enzyme treated soybean meal (ETS), and an untreated soybean meal with a prebiotic (USP). The chemical composition of protein sources used in this study are presented in Table 1. For each of the soy diets, US, ETS and USP were included at 25%, replacing fish meal, the diet formulation and nutritional composition of each diet are presented in Table 2. The enzyme treated soybean for the ES diet was produced by treatment with a 50:50 blend of Hostazyme X (main activity: Endo – 1,4 – β-xylanase, side enzyme activity: End – 1,4 -β-glucanase(cellulase), endo – 1,3(4) – β-glucanase, α-amylase, protease) and Hostazyme C (main activity; End-1,4 – β-glucanase, side enzyme activity: Endo – 1,4 – β-xylanase, endo – 1,3(4) – β-glucanase, α-amylase). The enzyme preparation was blended in 50°C water. The solution was added to 2L of water and 1kg of soybean meal and mixed for 15 minutes. The mix was then incubated for 3 hours at 50°C and manually mixed every 30 minutes. Following incubation, excess water was removed through pressing. The material was then further dried for 80°C. The diets were produced by cold pelletisation at Matís ohf. (Iceland). All dry ingredients were milled to homogenise particle size (IPHARMACHINE, Germany). Dry ingredients were homogenised in a standard food mixer (KitchenAid, USA) and the mix was milled again to improve the homogeneity of the feed. The dry mix was returned to the food mixer and fish oil was added and 200ml of water to produce the optimal consistency for processing in a pasta machine set to 0.5mm strings (ADE, Germany). Strings were dried in a commercial food dryer (Kreuzmayr, Austria) to <10% moisture content. During the 70-day feeding trial, fish were fed continuously by electric belt-feed with identical feed volumes at 15% excess fed based on the feed requirements for this developmental stage.

Table 1. Chemical composition of the protein sources used in feed treatments for this study.

Composition ($g\ kg^{-1}$)	*Fish meal*	*Wheat Gluten Meal*	*Enzyme Treated Soy*	*Untreated Soy (1)*	*Untreated Soy (2)*
Dry Matter	909	929	925	926	809
Crude Protein	659	742	482	487	479
Crude Lipid	107	16	5	9	4
Ash	139	11	73	68	62
Essential Amino Acids ($g\ kg^{-1}$)					
Arginine	40.3	25.0	35.7	34.7	35.1
Histidine	15.3	14.0	13.0	12.4	12.4
Isoleucine	26.0	24.5	22.7	21.4	22.0
Leucine	47.4	48.3	38.6	36.6	36.9
Lysine	51.2	11.4	31.8	30.0	29.7
Methionine	16.8	11.8	6.2	5.9	6.5
Phenylalanine	24.8	35.7	25.8	24.5	26.0
Threonine	28.3	17.8	21.6	20.6	21.8
Valine	32.8	27.4	23.9	22.7	22.5
Non-Essential Amino Acids ($g\ kg^{-1}$)					
Alanine	41.0	18.1	21.8	20.9	21.2
Aspartic acid	59.6	21.9	57.7	55.9	56.0
Glycine	43.6	23.3	21.2	20.5	20.3
Glutamic acid	83.9	260.0	93.6	88.3	87.9
Cystein + Cysteine	5.8	15.5	6.4	6.4	6.5
Tyrosine	19.2	22.9	18.1	17.6	18.1
Proline	28.7	86.9	25.2	25.0	23.8

Table 2. Feed formulation and chemical composition for feed treatments of this study.

	FM	ETS	US	USP
Formulation ($g\ kg^{-1}$)				
Fish Meal [a]	702.5	422.6	420.1	420.1
Pre-gelatinised Wheat [b]	189.8	87.8	90.9	90.9
Vitamin-Mineral Premix [c]	10.0	10.0	10.0	10.0
Fish Oil [a]	97.8	129.7	128.9	128.9
Wheat Gluten Meal	0.00	100.0	100.0	100.0
Enzyme Treated Soy Meal	0.00	250.0	0.00	0.00
Untreated Soy Meal (1)	0.00	0.00	250.0	0.00
Untreated Soy Meal (2)	0.00	0.00	0.00	250.0
Analysed Content,($g\ kg^{-1}$)	(%)	(%)	(%)	(%)
Dry Matter (water)	954	918	934	952
Crude Protein	505	485	493	501
Crude Lipid	175	170	172	175
Ash	120	93	95	83

2.3 Growth performance

The fish were not fed for 24hours prior to measuring. After 70-days of feeding, all individual fish from each treatment and replicate tank were anesthetised (phenoxyethanol 300ppm) and their wet weight (g) and total length (cm) measured. The specific growth rate (SGR) (%) over the study period was calculated: SGR = ((Ln(Final Weight)-Ln(Initial Weight)) x 100)/*t*, where *t* is the number of days the feeding trial was run. Mortality was monitored daily during the trial period.

2.4 Gut sampling

Following final measurements for growth performance, all fish were left for one week to recover and maintained on their respective experimental diets. Fish were then fasted for 12-hours. Three fish per tank n = 9 per treatment were randomly selected for gut microbiome analysis. Fish were humanely euthanised with a lethal dose of anaesthetic (2-phenoxyethanol 600ppm). The outside of the fish was washed in 90% ethanol followed by sterile distilled water. The gastro-intestinal (GI) tract and contents (from the start of the mid-gut, just below the pyloric caeca to the end of the distal gut) was sampled under sterile conditions and directly frozen at -80°C.

2.5 DNA extraction, PCR amplification and sequencing

GI tract samples were individually manually homogenised in a sterile petri dish with a sterile scalpel to physically break up the material. Sample material was transferred to a sterile 2ml Eppendorf tube with 300µl of sterile 1mm diameter silica beads (BioSpec Products, United States) and 800µl of CD1 solution from the QIAamp PowerFecal Pro DNA kit (QIAGEN, Germany). Samples were vortexed for 5 seconds and shaken at maximum speed (30Hz) in a laboratory mixer mill (Retsch MM400) for 1 minute. The supernatant (~800µl) was transferred to the PowerBead Pro tube from the QIAGEN QIAamp PowerFecal Pro DNA kit. The protocol for this DNA extraction kit was then followed and final DNA was eluted with 80µl of C6 solution. A DNA negative (no material added) was also run to ensure no contamination occurred during DNA extraction. DNA concentration was measured for each sample with 2µl with Invitrogen Qubit dsDNA BR Assay kit (Invitrogen, Carlsbad, CA, USA). DNA was diluted to 4ng μl^{-1} in 50µl aliquots. Samples were subjected to PCR of the V3-V4 regions of the 16S rRNA gene with a universal bacterial primer pair S-D-Bact-0341-b-S-17 (5′−CCTACGGGNGGCWGCAG-3′)/S-D-Bact-0785-a-A-21(5′-GACT- ACHVGGGTATCTAATCC-3′) (Klindworth et al., 2013). PCR master mix included diluted DNA, nuclease-free water, Q5 High Fidelity DNA polymerase (New England Biolabs, Ipswich, USA), Q5 GC Enhancer, 0.5µM of each primer containing Illumina overhand adapters, and 1 x Q5 Reaction buffer, 200µM dNTPs (New England Biolabs, Ipswich, USA). Both positive and negative samples were also run in the PCR to monitor for absence of contamination and successful amplification. The thermocycling protocol had an initial denaturation step

(90°C for 30s), followed by 35 cycles of: denaturation (90°C for 10s), annealing (52°C for 30s), and extension step (72°C for 30s), with a final extension (72°C for 2 min). Libraries were multiplexed with Nextera XT v2 barcodes (Illumina, USA), normalised using *Sequel-Prep* normalisation places (ThermoFisher Scientific, USA) and sequenced on MiSeq desktop sequencer (Illumina, USA) using v3 chemistry and 2 x 300 cycles.

2.6 Behaviour

Following gut microbiome sampling, eight (8) fish from the remaining tank population were randomly selected to be uniquely tagged with Visible Implant Elastomer (VIE) colour tags (Leblanc and Noakes, 2012) under anaesthetic (2-phenoxyethanol 300ppm). Each fish was injected parallel to the dorsal fin, below the surface skin layer with two 1cm strips of colour with unique combinations so each of the 8 selected fish per tank (n=24 per treatment) could be identified and followed through all behavioural observations. All remaining untagged fish per tank were retained in tank to maintain a consistent stocking density but were not included in behavioural observations. Each tagged fish was observed in two different behaviour contexts (a swimming test and an open field test with shelter) and each of these tests was performed two times. There was always a one-week recovery period between each behavioural observation. Behaviour observations were performed in a dedicated room to minimise external disturbance. Fish were individually tested in all behavioural observations. Behaviour was recorded using a monochrome camera (Basler Ace acA1920-150um, Germany) with a frame rate of 30Hz and resolution of 1280 x 1024 pixels placed 112cm (swimming test arena) and 110cm (open-field test) above the water level of the respective behavioural arena. Video recordings were analysed with EthoVision XT software (Noldus, The Netherlands), which was used to track fish in each arena. The VIE tags were recorded for each fish after each behaviour observation so their individual data could be tracked across the different time and contexts of the behavioural observations.

2.6.1 Swimming test for assessing response to stress and exploration

A swimming test was used to collect behaviour observation data for stress response and the behaviour trait of exploration. The apparatus for assessing these traits consisted of 4 circular arenas (diameter = 25cm, water depth 7.8cm (4L), height= 15cm). The arena was illuminated from below to provide uniform light intensity (260 lux) Figure 1. Tagged fish from each tank were collected from the trial tank and transferred in identical white transfer buckets with closed tops and then placed into individual arenas in random order. All four arenas were filmed simultaneous and continuously for 20 minutes and for the purposes of analysis was separated into four virtual periods. The 0-5 minutes was the acclimation period (AC), 5-10 minutes was the normal swimming period (NS). At the 10-minute mark a stress was elicited (bottom lighting was switched off for 3 seconds and then switched back on again), minutes 10-15 was thus the post light stress period (PLS), and 15-20 minutes was the recovery period (RC). Behaviour was not recorded while the light was off.

Variables collected through the swimming tests were, mean distance from centre-point (cm): the distance of the centre-point of the fish body from arena centre (DisCent). Total distance moved (cm): the distance travelled by a fish measured from the centre-point of body between consecutive X-Y coordinates (TotDis). Mean velocity (BL s^{-1}): the distance moved by the centre-point of body per unit time between consecutive X-Y coordinates normalised to body lengths per second (Vel). Absolute angular velocity (° s^{-1}): expressed in degrees per second was calculated by EthoVision XT software. Vangn = RTAn/tn – (tn-1), where RTAn represents the relative turn angle for sample n, and tn-(tn-1) is the time difference between the current and previous sample (AngVel). Mobility state, the cumulative duration for which the fish body is changing, highly mobile (s) when cumulative duration is 60% (HiMob). Moderate mobile (s) when cumulative duration was between 20-60% (MedMob). Immobile (s) when cumulative duration was below 20% (Immob).

2.6.2 Open-field test for assessing boldness

An open-field test (OFT) with a shelter was used to assess boldness (Toms et al., 2010). The apparatus for assessing this trait consisted of 4 rectangular arenas (40 x 30 x 25 cm), water depth 6cm (7L) with an identical shelter placed in the bottom-right of each arena (14 x 6.5 x 6.5 cm). The arena was illuminated from below to provide uniform light intensity (260 lux) Figure 2A. Tagged fish from each tank were collected from the trial tank and transferred in identical white transfer buckets with closed tops and then placed in random order into individual arena shelters through a top compartment (4cm diameter) which was then closed, and the main shelter door (a sliding opaque trapdoor) was kept shut, closing off access to the arena. All four arenas were filmed simultaneous and continuously for 25 minutes. The first 5 minutes the fish remained shut inside the shelter acting as an acclimation period, after which the door to each arena shelter was lifted simultaneously and filming continued for a further 20minutes uninterrupted. Each individual arena was virtually divided into four zones using the EthoVision XT software. These zones were called Shelter, Entry, Border and Centre (Figure 2B). The centre zone is considered high-risk and staying close to the border edges of a space is considered more cautious and an indicator of a shyer individual (Benhaïm et al., 2020; Dahlbom et al., 2011). Variables used to characterise OFT behaviour for the trait of boldness were as follows: Latency (s) to emerge-time taken to exit the shelter (Lat). Time spent in each zone (s) (Shelter, Entry, Border, Centre) respectively (Shelt, Ent, Bord, Cent). Mean distance from shelter (cm) (DisShelt). Number of returns to shelter (Ret). Cumulative duration spent highly mobile (s) (HiMob). Distance moved: the distance travelled by a fish measured from the centre-point of body between consecutive X-Y coordinates. Mean velocity (BL s^{-1}) Absolute angular velocity (° s^{-1}).

Figure 1: Swimming test arena used for observation of the behavioural trait of exploration and to determine stress response.

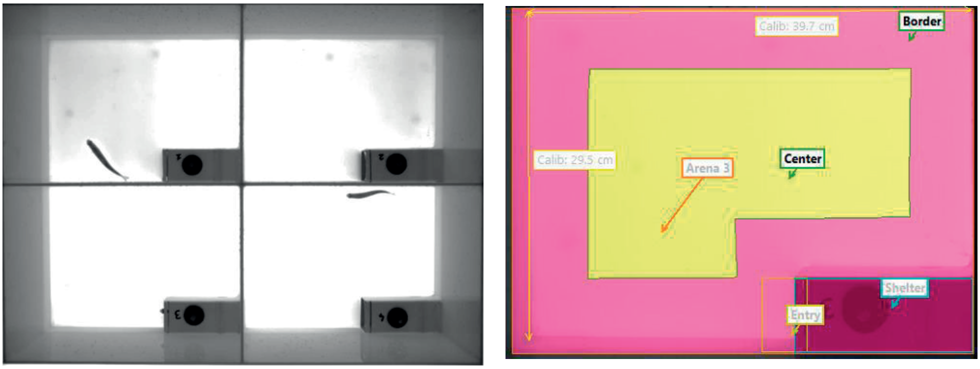

Figure 2: A. Open Field test arena used for observation of the behavioural trait of boldness. **B.** An arena with four virtual zones outlined in the EthoVision XT software, Shelter, Entry, Border, and Centre.

2.7 Statistical methods

Statistical analyses were performed in Rstudio version 3.6.1 (2019-07-05). All tests were two-tailed with a significance level set to α = 0.05.

2.7.1 Growth performance

SGR (%) was used to assess growth performance across the different feed treatments in this study. A General Linear Mixed Model (GLMM) with the package *lme4* (Bates et al., 2015) was selected defining feed treatment (FM, US, ETS, USP) as a fixed factor and tank (1,2,) as a random nested factor of feed treatment. Since the random nested factor of tank did not cause significant variation in SGR (%), a simplified statistical test was adopted. A Linear Model (LM) with the package *nmle* (Pinheiro et al., 2020), where feed treatment was a fixed factor and the model residuals had a normal distribution. A Tukey post-hoc test was applied.

2.7.2 Gut microbiome

To assess the gut microbiome of fish fed different dietary treatments in this study, demultiplexed FASTQ files from Illumina were processed to produce amplicon sequence variants (ASVs) with DADA2 package version 1.16.0 (Callahan et al., 2016). The function filterAndTrim set variables as follows: truncLen=c(280,250), trimLeft=21, maxN=0, maxEE=c(2,2), truncQ=2. Taxonomy was assigned to ASVs from version 138 of the SILVA database and the function *assignTaxonomy* (Quast et al., 2013). The R packages, *phyloseq* (McMurdie and Holmes, 2013), *microbiome* (Lahti and Shetty, 2017) and *vegan* (Oksanen et al., 2020) were used to analyse the microbiome community and ggplot2 was sued to visualise key data (Wickham., 2016). The average read number output from the DADA2 pipeline was 20643±12702, and two samples were removed as they did not contain any reads after processing. Five PCR negative samples were also sequenced to control for any contamination that may have occurred during sample amplification. These controls were used to remove suspected contamination from samples using the *decontam* package with prevalence method and threshold of 0.5 (Davis et al., 2017). Read depth was normalised across all samples using the function *rarefy_even_depth*. Raw 16S rRNA gene amplicon reads can be found in the Sequence Read Archive following submission.

Alpha and beta diversity indices were used to quantitatively analyse the gut microbiome. Alpha diversity measures selected were, observed richness of AVSs, Shannon diversity, Chao1 diversity and Pielou´s evenness. A GLMM was used to assess if there was a significant in the alpha diversity measures between the different feed treatments in this study. In this model, feed treatment was a fixed factor and tank was a random nested factor of feed treatment (which was tested by a Likelihood Ratio Test (Fox et al., 2011)). A Tukey test was applied for post-hoc testing. Beta diversity was assessed by transforming microbiome community data using a Bray-Curtis dissimilarity matrix. An Analysis of Similarity (ANOSIM) was performed to determine if there is a significant difference between and within the gut microbiome community of fish fed different diets. Relative abundance of taxa at the phylum level and genera level as a proportion was calculated. Taxa present in the community at <1% relative abundance were grouped into a category labelled “Other”.

2.7.3 Behavioural characteristics

To assess stress response, all swimming test variables were analysed using GLMM. For each analysis, the explanatory variables included in the full model were, feed treatment (FM, US, ETS, USP), period (AC, NS, PLS, RC), and TL, and interactions between feed treatment and period were considered. Random effects considered in the model were, and trial replicate number (1, 2), Tank (1,2,3) nested in feed treatment, and individual.

To assess the behaviour trait of exploration, a Principal Component Analysis (PCA) was applied to all variables measured for the period NS. A correlation matrix was used to verify multi-collinearity between variables, and those variables that were highly correlated were removed. The remaining variables were collapsed into first principal component (PC1) scores, which explained much of the variation in the data. To assess the behaviour trait of boldness, the same method as for exploration as applied on the variables collected during the OFT. The scores for each trait were then compared between feed treatments in two respective GLMM models where score was the response variable. The fixed factors each of these models were, the feed treatment, and the total length TL (since size may influence behaviour traits scores). The random factors of the model were, trial replicate number (1, 2), Tank which was a random nested factor of feed treatment and individual was also considered as a random factor.

2.7.4 Repeatability of behaviour traits

For exploration score, and boldness score, a repeatability model was applied using the Rstudio package *rpt* to investigate if each respective trait was repeatable at the individual level across all feed treatments i.e., personality traits, between the first and second repeat of the test. The parameters nboot and npermut were set at 1000.

2.7.5 Behavioural syndrome

To assess if fish in this trial displayed a behavioural syndrome, a GLMM test was run to determine if there was a relationship between the two scores (boldness ~ exploration) for each of the feed treatments respectively (FM, US, ETS, USP). The fixed factor of this model was TL and random factors were the trial number (1,2) and individual.

3 Results

3.1 Growth performance

There was a significant difference in SGR% between dietary treatments in this study Figure 3. FM fed fish had significantly higher SGR% than fish fed US. The enhanced soy diets, ETS and USP fed fish had slightly higher SGR% on average than the conventional, US fed fish, but the values were statistically comparable to both FM and US.

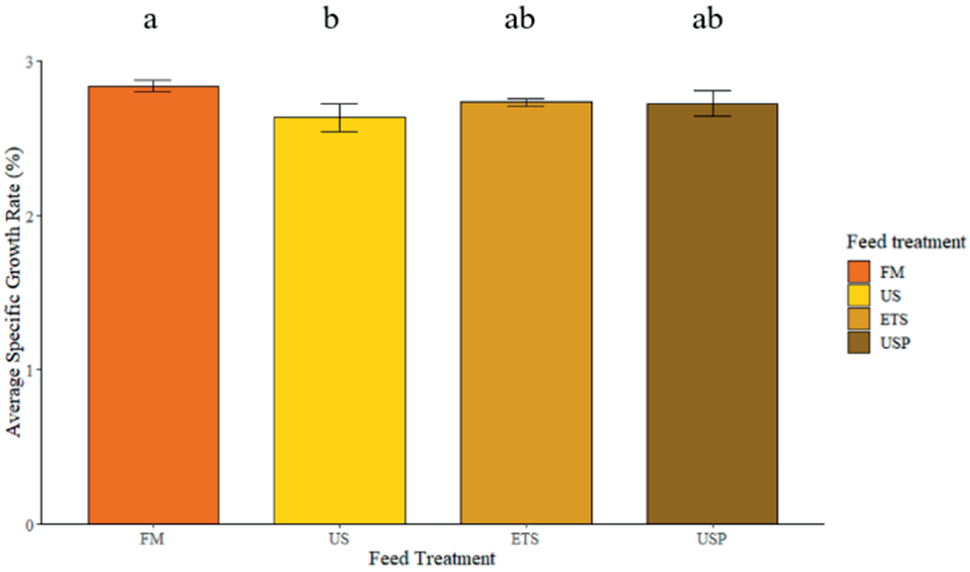

Figure 3: Average Individual Specific Growth Rate (%) for each dietary treatment.

3.2 Gut microbiome

There was a significant difference in the gut microbiome community alpha diversity measures for Shannon diversity (Figure 4B) and for Pielou evenness (Figure 4D), but not for the observed richness of ASVs (Figure 4A) or Chao1 diversity (Figure 4C) between the dietary treatments. For both Shannon diversity and Pielou´s evenness, the highest community diversity and evenness values were observed for the FM diet, which was significantly higher than the values for all other treatments, followed by the unenhanced US diet which had comparable Shannon diversity and evenness to the enhanced USP fed fish. The enhanced ETS diet had significantly lower gut microbiome community Shannon diversity and evenness than fish fed any other diet.

The gut microbiome communities were significantly different between feed treatments, and there was a much greater similarity between individual fish from the same feed treatment than between individuals from different feed treatment (ANOSIM P = 0.001, R=0.84). The NMDS plot (Figure 5) shows distinct clustering by feed treatment.

At the phylum level, all feed treatments in the study the phyla with the largest proportion presence are *Firmicutes* (FM=0.81±0.1, US=0.93±0.03, ETS=0.99±0.01, USP=0.95±0.03), followed by lower proportions of *Actinobacteria* (FM=0.1±0.06, US=0.05±0.03, ETS=0.01±0.01, USP=0.03±0.01) and *Proteobacteria* (FM=0.09±0.12, US=0.01±0.01, ETS=0.01±0.01, USP=0.02±0.03) which were at similar levels.

At the taxonomic level of genus, there were nine genera with a relative abundance >1% of the community (Figure 6), all other genera were present at very low relative abundance. The genera composition between the FM feed treatment and the three feed treatments containing soybean meal showed distinct difference. The genera, *Anaerosalibacter*, *Clostridium_sensu_stricto_18*, *Clostridium_sensu_stricto_7*, *Hathewaya* and *Peptosteptococcus* all had a greater relative abundance in FM fed fish gut microbiome than US, ETS or USP fed fish gut microbiomes. In all these same genera however, there was a trend that relative abundance was higher in US and USP than the proportion observed in ETS fish gut microbiome. The LABs, *Leuconostoc* and *Weissella* had the lowest relative abundance in FM fish gut microbiomes, slightly higher in the ETS fish gut microbiomes and highest in the US and USP fish gut microbiomes. For the LAB *Enterococcus* there was a differing trend, the relative abundance was very high in the ETS fish gut microbiome, low but present in the FM and USP fish gut microbiome and absent in the US fish gut microbiome. *Staphylococcus* was observed in similar relative abundance for FM, US and USP fish gut microbiomes but much lower in ETS fish gut microbiome.

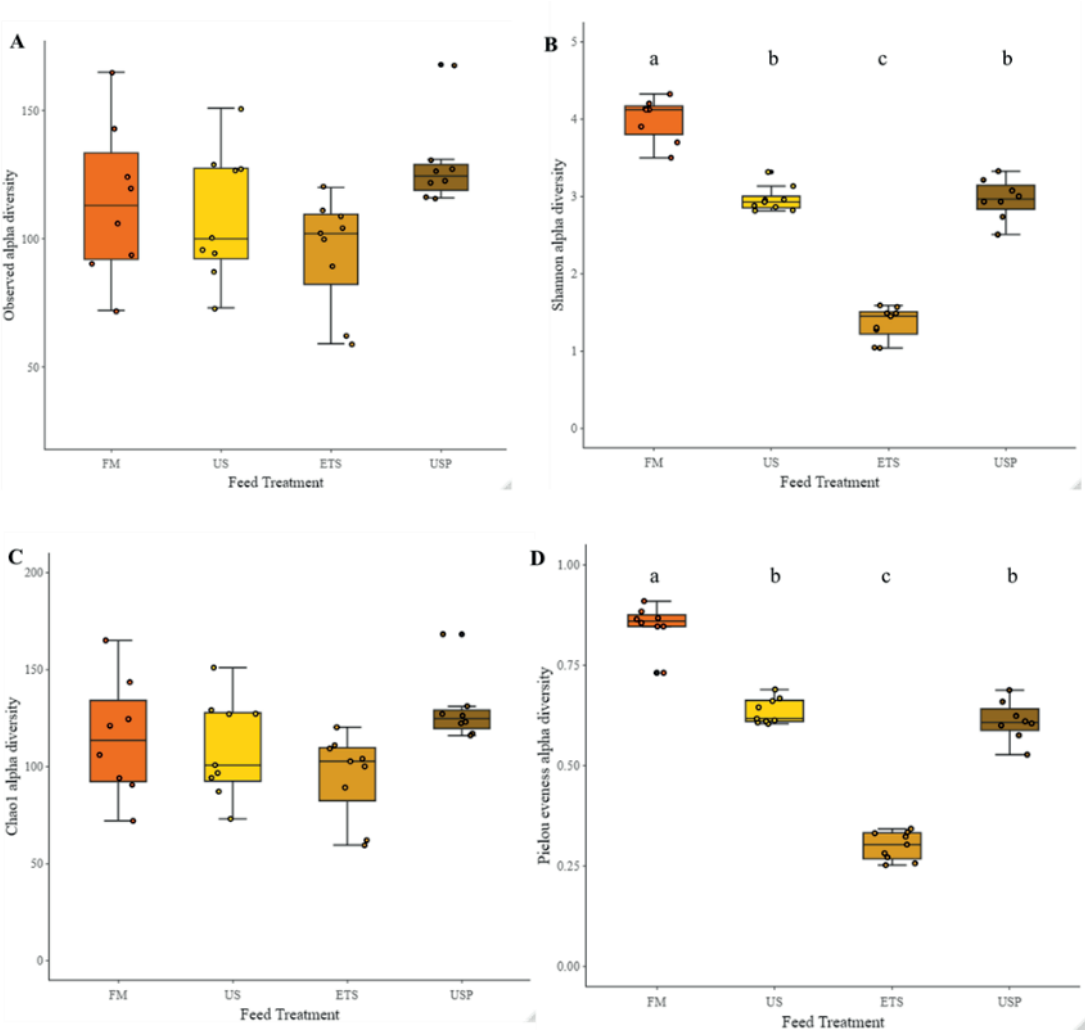

Figure 4: Alpha diversity measures of fish fed different dietary treatments: (**A**) observed richness of ASVs, (**B**) Shannon diversity, (**C**) Chao1 diversity, (**D**) Pielou evenness.

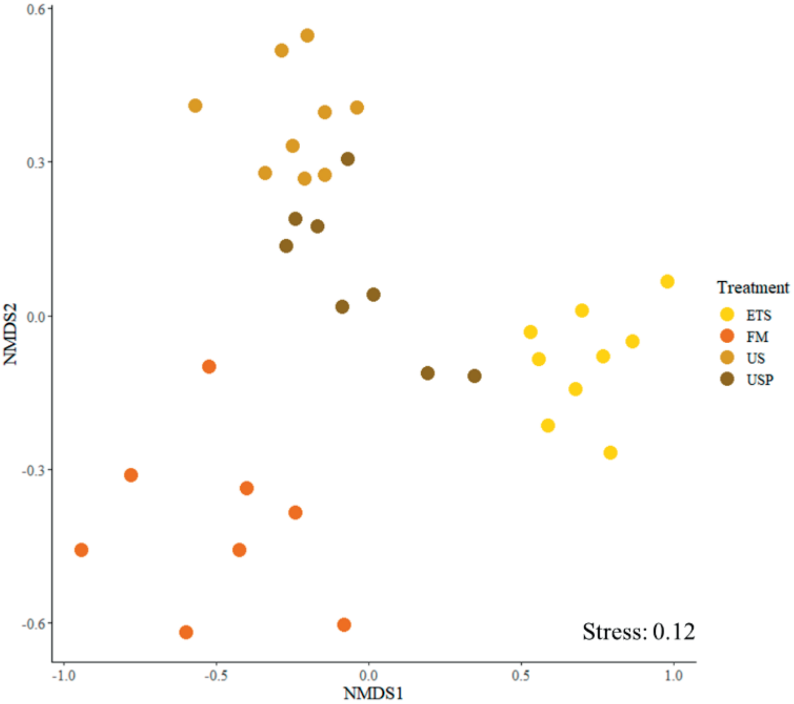

Figure 5: NMDS plot of individual fish gut microbiome composition for each feed treatment.

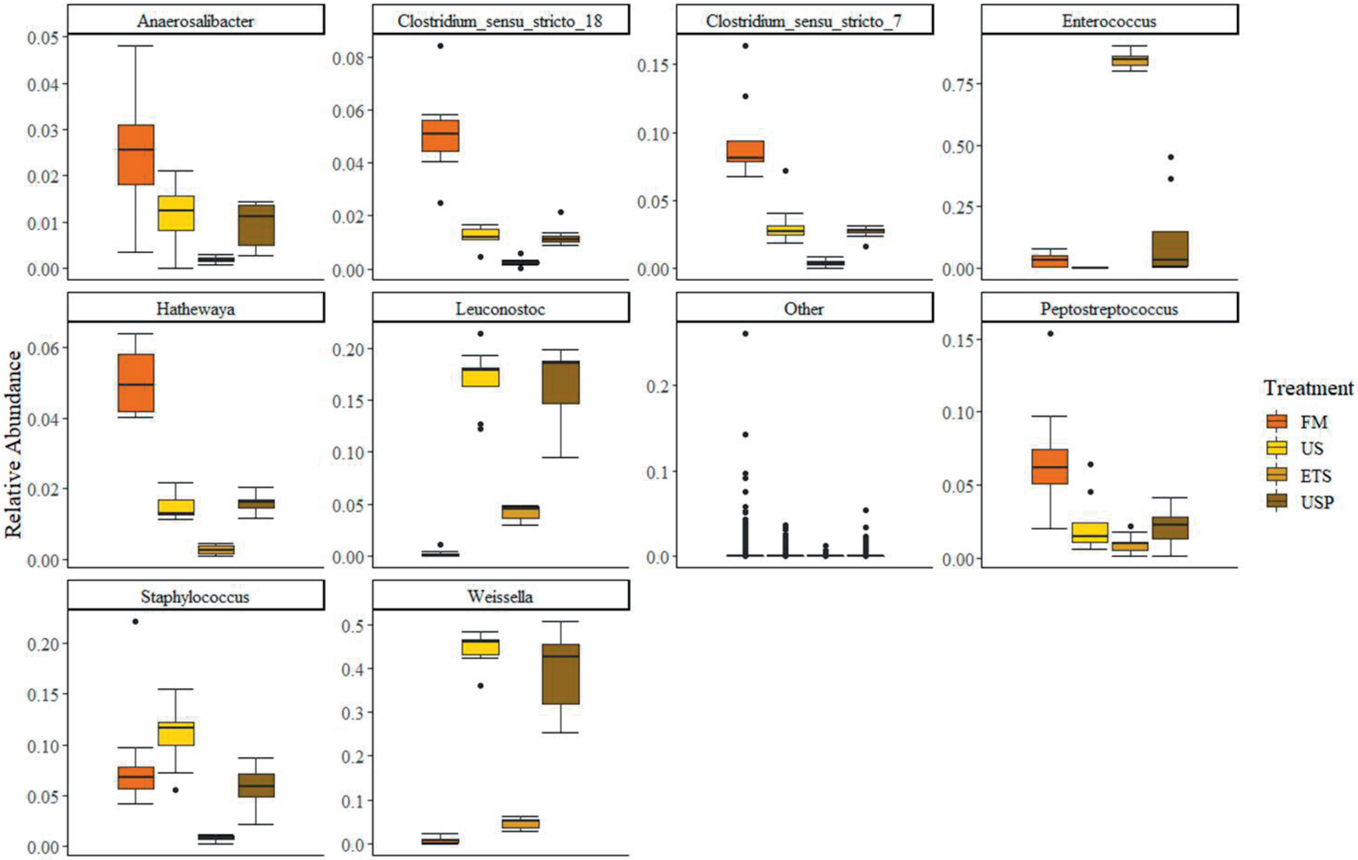

Figure 6: Relative abundance of genera present in gut microbiome community of fish fed each dietary treatment.

3.3 Behavioural characteristics

3.3.1 Swimming Test

There was no significant difference in any of the swimming variables between any of the feed treatments. There was a significant difference between period for all variables except High Mobility (s) (Supplementary Figure 1) but this did not appear to be driven by the elicited light stress but instead by time spent in the arena. The interaction between feed treatment and period was significant for AngVel (° s^{-1}) for USP feed during PLS period (χ_2=17.53, df=9, p=0.03), Vel (BL s^{-1}) for US feed during RC period (χ_2=17.54, df=9, p=0.041), MedMob (s) for USP in PLS and NS (χ_2=19.17, df=9, p=0.024), and Immob (s) for USP for PLS and NS (χ_2=18.83, df=9, p=0.03). There was a significant effect of swimming test replicate (1,2) for TotDis (cm), DisCent (cm), HiMob (s), and MedMob (s). For all variables there was a significant effect of TL (cm), for the variables DisCent (cm), TotDis (cm), Vel (BL s^{-1}), HiMob (s), and MedMob (s) there was a negative relationship with TL (cm) (larger fish had lower values for these variables), and for AngVel (° s^{-1}), and Immob (s) there was a positive relationship with TL (cm) (larger fish had higher values for these variables).

For the trait of exploration, PC1 explained 70% of variation in the data. For each feed treatment EP score was significantly repeatable across the two test replicates (Table 3). For the PC1 EP score (Figure 7), a high value indicates greater angular velocity (° s^{-1}) (loading 0.33) and longer time spend immobile (s) (loading = 0.43) and low values indicates greater total distance travelled (cm) (loading = -0.42), higher swimming velocity (BL s^{-1}) (loading = -0.44), greater average distance from arena (cm) (loading = -0.3), greater time spent highly mobile (s) (loading = -0.26) and greater time spent mobile (s) (loading= -0.42). PC1 EP score therefore presents a gradient from more exploratory (low values), to less exploratory (high values). There was no significant difference in the EP score between the different feed treatments, although there is a trend visible showing FM fed fish tended to be more exploratory than fish fed any of the soy diets, and the ETS fed fish tended to be the most exploratory of any of the soy fed fish. The random factor of test repeat number was not significant, but the random factors of individual and tank had a random effect, and the fixed factor of total length (cm) was significant (χ^2=281.15, df = 1, p=<0.001)

Table 3. Repeatability results for the behaviour traits of exploration.

Exploration	**R**	**CI**	**P value**
FM	0.909	0.807, 0.961	<0.001
US	0.741	0.524, 0.882	<0.001
ETS	0.805	0.643, 0.912	<0.001
USP	0.665	0.41, 0.847	<0.001

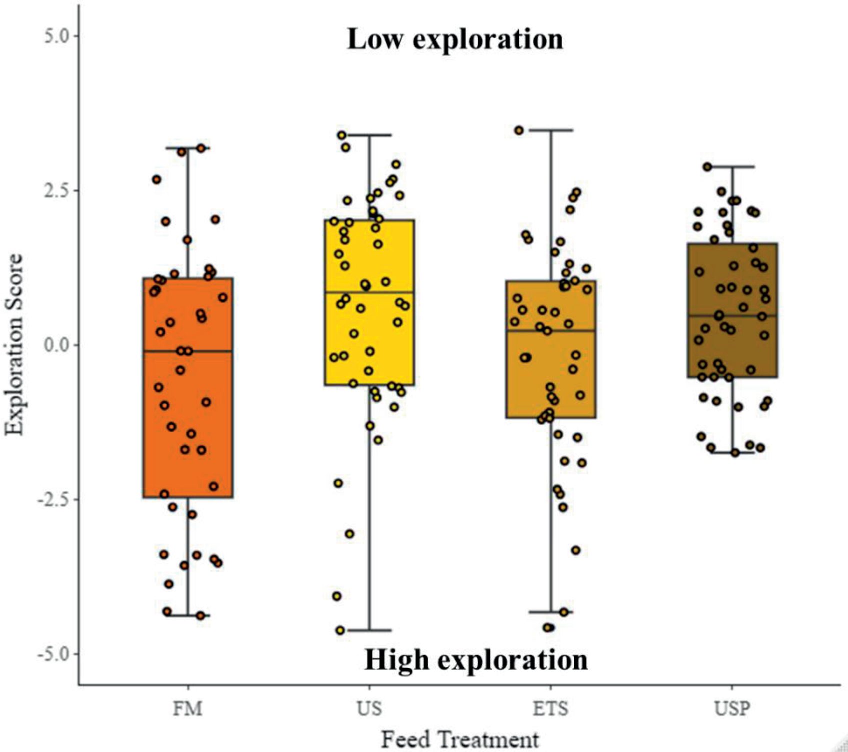

Figure 7. Exploration score of fish fed different feed treatments where lower values indicate high exploration and high values indicator low exploration.

3.3.2 Open Field Test

For the trait of boldness, PC1 explained 48% of variation in the data. For each feed treatment the boldness score was significantly repeatable across the two replicate tests (Table 4). For the PC1 boldness score (Figure 8), a high value indicates a greater total distance travelled (cm) (loading = 0.43), higher swimming velocity (BL s^{-1}) (loading = 0.41), greater number of returns to the shelter (loading = 0.3), greater time spent in the entry zone (s) (loading = 0.12), greater time spent in the border zone (s) (loading = 0.41), greater time in the centre zone (s) (loading = 0.21), and higher mobility (s) (loading = 0.31). A low value indicates, greater time spent in the shelter (s) (loading = -0.35), Latency to exit shelter (s) (loading = -0.25) and greater absolute angular velocity (° s^{-1}) (loading = -0.21). PC1 boldness score therefore presents a gradient from shyer (low values) to bolder (high values). There was no significant difference in the boldness score between feed treatments, although there is a trend visible showing FM fed fish tended to be bolder than fish fed any of the soy diets, and the US fed fish tended to be shyer than any other fish. The random factor of test repeat number was not significant, but the random factors of individual and tank had a random effect, and the fixed factor of total length (cm) was significant (P=<0.001, df=1, χ^2=31.92 S.E =0.11, Estimate= -0.6).

Table 4. Repeatability results for the behaviour trait of boldness.

Boldness	**R**	**CI**	**P value**
FM	0.766	0.546, 0.896	<0.001
US	0.459	0.033, 0.775	0.015
ETS	0.594	0.259, 0.82	0.0013
USP	0.565	0.205, 0.823	0.0028

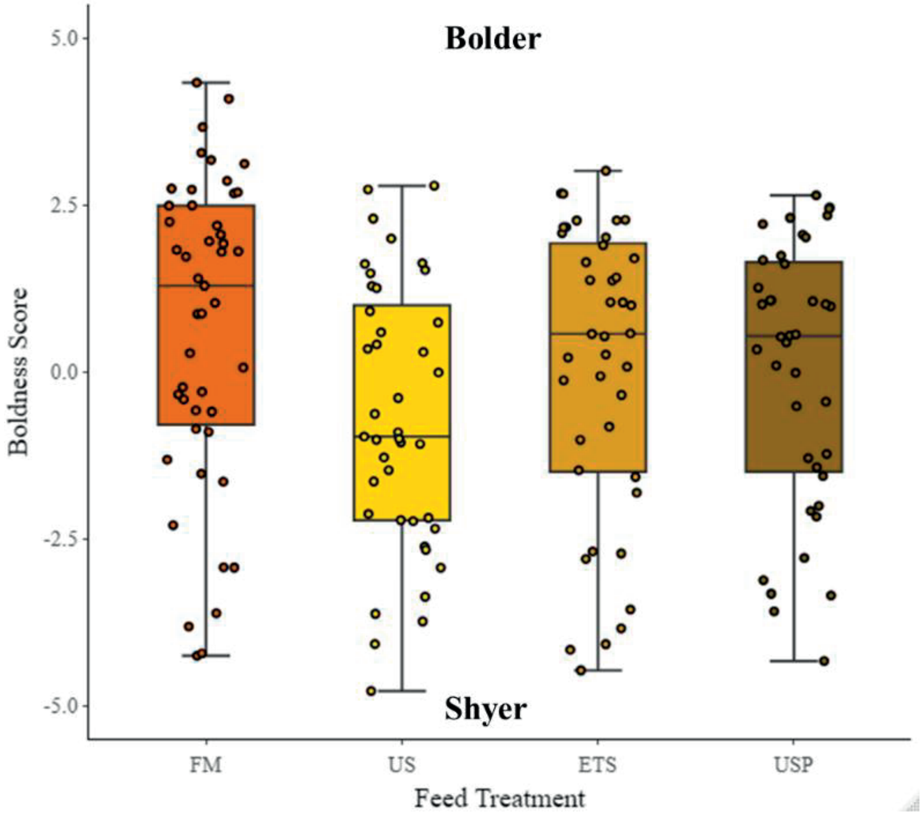

Figure 8. Boldness score of fish fed different feed treatments where lower values indicate shyer individuals and high values indicate bolder individuals.

3.3.3 Behaviour syndrome

There was a significant correlation between the exploration and boldness scores for fish fed FM (χ_2=4.52, df=1, p=0.03), USP (χ_2=4.32, df=1, p=0.038), and ETS (χ_2=10.15, df=1, p=0.001). There was no significant correlation between the two behavioural traits scores. The random factors did not affect the correlation.

4 Discussion

The objective of the present multi-disciplinary study was to optimise the application of soybean meal in formulated aquafeeds for salmonids. This study reports the impact of two novel enhancements of soybean meal (SBM), an enzyme pre-treated SBM (ETS), and a SBM with addition of the prebiotic, FOS (USP) compared with an untreated SBM (US) and a fish meal control (FM) on growth performance, gut microbiome, and individual behaviour traits relevant to the culture of juvenile Atlantic salmon (*Salmo salar*).

The growth performance of Atlantic salmon in this study across all treatments was within a normal range for the freshwater stage at the experiment temperature (Nathanailides et al., 1995; Sørensen et al., 2017). The findings are consistent with the existing literature that FM diets can support higher growth than untreated SBM for Atlantic salmon, especially at higher inclusion levels of 20% SBM (Romarheim et al., 2011b), and at 31% SBM (Storebakken et al., 1998). However, during the freshwater stage, salmon fed 16.7% (Sahlmann et al., 2015) and 40% (Øvrum Hansen et al., 2019) SBM performed comparably to FM diets. In seawater phase, dietary inclusion levels of 20% SBM and higher reduced growth compared to FM diets (A. Krogdahl et al., 2003), although variations exist in the literature (Olli et al., 1995). The two novel enhancements applied in this study supported comparable growth performance to FM, suggesting that both enzyme pre-treatment and addition of FOS have potential benefits to improve the use of SBM for salmon during freshwater stages. Similar improvements in growth performance have been seen when replacing 40% of protein from SBM with reduced content of oligosaccharides and ANFs with conventional SBM (Refstie et al., 1998). In Japanese seabass (*Lateolabrax japonicus*) NSP enzymes applied to feed also improved growth performance (Ai et al., 2007) and NSP targested enzyme treatment of *Gracilaria lemaneformis* enhanced growth perfromenace in white-spotted snapper (*Lutjanus stellatus*) (Zhu et al., 2016). Not all studies, however, found growth performance benefits of NSP-targeted enzyme treatment of SBM for Atlantic salmon (Denstadli et al., 2011). Furthermore, growth benefits were not seen with phytase pre-treated SPC in SW Atlantic salmon (Denstadli et al., 2007). The addition of FOS can be beneficial for Rainbow trout growth performance (Ortiz et al., 2013), however, when FOS was added to FM diets for Atlantic salmon, improvement in growth performance have not been observed (Grisdale-Helland et al., 2008).

The differences in the gut microbiome community assemblage between fish fed different diets have been observed in many studies for salmonids, between FM and SBM (Gajardo et al., 2017; Michl et al., 2017b) and between different treatments of conventional proteins (Catalán et al., 2017; Green et al., 2013b). Existing studies have also found FM diets to support greater diversity and community evenness in fish gut microbiome (Bruce et al., 2018; Reveco et al., 2014). The findings of this study that USP fed fish had comparable diversity to US, and that ETS fed fish had lower diversity than all other treatments, were similarly found in white sea bream (*Diplodus sargus*) fed FOS (Guerreiro et al., 2018). However, existing research observed increased gut microbiome diversity measured with the additions of prebiotics for juvenile hybrid Tilapia (*Oreochromis niloticus*♀ × *Oreochromis aureus* ♂) (Liu et al., 2017). The lack of difference in the present study may be driven by the need for longer term continuous administration to detect positive gut health impacts of feed additives in fish (Burgos et al., 2018). It has been noted that diets with SBM which contains natural oligosaccharide sources may mask any beneficial effects of additional prebiotics (Gibson and Roberfroid, 1995; Guerreiro et al., 2018). The impact of low values for diversity and evenness found for ETS fed fish will be important to elucidate, since high gut microbiome community evenness and diversity values have been associated better productivity in aquaculture (Infante-Villamil et al., 2021). In the case of this study, it seems that this lower diversity and evenness is driven by a dominance of the lactic acid bacteria (LAB) *Enterococcus*, a genus that has been associated with growth and immune benefits to fish (Alshammari et al., 2019) and since the presence of this genus has not excluded other taxa establishing in the gut, then the lower evenness and diversity may not adversely affect fish gut health. Although community dominance at such an early development stage should be monitored to ensure that an undesirable dysbiosis does not establish itself (Perry et al., 2020). This study suggests that the application of FOS has quite different impacts than the enzyme-treatment at the genus taxonomic level, both may be having a prebiotic effect, but stimulate the growth of different communities. The increased presence of the LABs in SBM diets compared to FM are consistent with the existing literature for salmonids (Gajardo et al., 2017), as is the addition of prebiotic ingredients increasing levels of LAB in the common carp (*Cyprinus carpio*) (Momeni-Moghaddam et al., 2015). LABs have been associated with improved digestive function, and gut health, and disease resistance (De Vuyst and Leroy, 2007; Ringø et al., 2020, 2018). Since the levels are lower for these two taxa in the ETS fed fish, it suggests that the enzyme treatment may have broken down part of the SBM favoured by these two taxa, a phenomenon that warrants further investigation. The greater presence of *Clostridium_senu_stricto_7*, *Clostridium_senu_stricto_18* and *Peptostreptococcus* in FM fed fish have been found in faster growing individual Rainbow trout (Chapagain et al., 2019), with is consistent with the growth performance findings for this trial. These bacteria have been linked to fermentation of different amino acids (Neis et al., 2015) suggesting their growth may be facilitated my amino acids present in FM that are not present or less present in SBM. The prevalence of the taxa *Hathewaya* and *Anaerosalibacter* in FM fed fish compared with SBM in the study, have also been found in Chinook salmon (*Oncorhynchus tshawytscha*) gut microbiomes associated with presence of FM (Steiner et al., 2021). There is a need for future research to address the functional role of LAB for Atlantic salmon and to establish taxa associated with growth performance and gut health benefits to improve the application of feed additives and dietary enhancements.

This study is the first to investigate behavioural traits relevant to welfare under different protein and protein enhancements for Atlantic salmon. While the scores the traits of exploration and boldness behaviour traits did not differ significantly between the diets in this study, there was an interesting trend that FM fed fish displayed a more proactive coping strategy than fish fed any of the SBM diets. For ETS fed fish, there was indication of more proactive-type behaviour compared to US fed fish, displayed in the swimming test by the lower thigmotaxis (staying close to the border of an arena) shown in the former fish (Benhaïm et al., 2020). The high level of repeatability of both traits may show consistent freedom to express natural behaviour which may indicate a good welfare condition (Huntingford and Adams, 2005; Webster, 2001) and for both traits this was highest in FM fed fish indicating they may be most suited with the aquaculture environment. Furthermore, existing studies have indicated that more proactive individuals do show a higher degree of repeatability for behaviour traits which matches well with the findings of the present study The light stress did not appear to produce a response in the fish tested, suggesting the need for a different stress source, as the fish may have been habituated to light stress as they were kept under continuous light (Madaro et al., 2016). These results are consistent with results from juvenile Rainbow trout, when fish were fed a plant-based diet, there was an increase in apathetic behaviour and an increased stress response, (both traits of reactive coping styles (Castanheira et al., 2017)) compared to a marine diet (Sadoul et al., 2016). Furthermore, in juvenile Senegalese sole (*Solea senegalensis*) individuals fed diets enriched with fish oil from Cod liver, compared with plant oils from linseed, soybean and olive, were more proactive (Ibarra-Zatarain et al., 2015). Furthermore, studies have shown that gut-altering substances can alter swimming behaviour, such as feeding the probiotic, *Lactobacillus rhamnosus* IMC 501 to Zebrafish (*Danio rerio*), which altered shoaling behaviour (Borrelli et al., 2016). Fish with proactive coping styles, have been noted to have, higher reproductive success in gilthead seabream (*Sparus aurata*) (Ibarra-Zatarain et al., 2019), higher growth rates for salmon (Damsgård et al., 2019), feed more rapidly after stress (Øverli et al., 2007) and are less sensitive to environmental stress (Höglund et al., 2008) all of which may make them better suited to the intensive farming environment of salmonid aquaculture (Huntingford and Adams, 2005) as these individuals may be more productive and also experience better welfare. It is possible that broodstock selection and process of domestication of Atlantic salmon genetic lines could already have selected for more proactive styles (Huntingford and Adams, 2005; Ruiz-Gomez et al., 2008) which may also explain why there was low responsiveness to the elicited stress in the present study.

In the present study behavioural syndrome detected for all feed treatments expect US fed fish, this indicates that US fed fish may be experiencing different conditions or pressures than the fish fed other diets (Sih et al., 2004). Domestication of salmon may also reduce the presence of behavioural syndromes, in a study of urban song sparrows no correlation between traits of boldness and aggression could be found, whereas there was a correlation, and thus behaviour syndrome in their wild counterparts (Evans et al., 2010) this also may suggest that the behaviour of US fed fish in this study was most impacted by the culture conditions and have adjusted by becoming more behaviourally plastic or showing adapted behaviours to cope (Sih et al., 2004). There is a need for further research attention on the impact of dietary proteins and treatments on behaviour and welfare for farmed Atlantic salmon to support the optimisation of existing feed materials and the industry uptake of alternative proteins. This study also highlights the need for greater understanding on how domestication may alter salmon behaviour and therefore welfare in intensive farms which will help inform selection programs to optimise coping styles in farmed fish (Baker et al., 2018; Ferrari et al., 2016).

In this study, FM fed fish grew the fastest, had a distinctly different gut microbiome community visible at the taxonomic level and tended to have more proactive behaviour and potentially better welfare compared to any of the SBM treatment in this study. Conversely, the US fish differed the most from the FM fish in all aspects of this multi-discipline approach, showing poorer growth, a lower diversity and evenness of the gut microbiome community and behaviours that indicated differing impact compared with any other diet. While enhancements of SBM ameliorated some of these traits, there were still difference with FM fish. These findings highlight the importance of such multi-disciplinary studies to elucidate the impact both to traditional productivity measures for aquaculture but also to measures that explore indirect impacts of nutrition on immune development and welfare. Future studies should try to establish functional links between these different fields to optimise Atlantic salmon aquaculture. This will be especially important as new feed enhancements and alternative proteins enter the market to ensure they are safe and effective for the aquaculture sector. This information with also inform improved breeding selection programs.

5 Conclusion

The results of this multi-disciplinary study suggest that the SBM enhancements evaluated provide growth performance benefits compared to unenhanced SBM, alter the gut microbiome community assemblage compared to both unenhanced SBM and FM and in the case of enzyme pre-treatment, support a strong dominance of the LAB *Enterococcus*. This study demonstrates for the first time the impact of SBM on juvenile Atlantic salmon behaviour traits, showing there is a trend that a plant-based diet may increase reactive coping styles. This may have adverse consequences for welfare of fish in intensive farm systems. The two enhancements of SBM offer some improvements to conventional SBM which provide a promising basis for continued investigation to optimise the application of this widely used protein source. Future studies should attempt to elucidate the functional link between nutritional composition of protein sources, composition of the gut microbiome and their influence on behaviour traits relevant to the aquaculture sector.

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7 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

8 Author Contributions

AL developed experimental design, supported feeding trial, and animal husbandry, and performed behaviour and microbiology lab work and data analysis and led the writing of this manuscript. CS managed feeding trial and animal husbandry and performed behaviour lab work and supported analysis. BB supported feeding trial and animal husbandry and supported behaviour lab work and analysis. WK supported experimental design, provided expert advice, and contributed to writing and editing this paper. SK provided expert advice and practical support in gut microbiome sample processing and analysis. SS provide advice and practical lab support for gut microbiome sample processing. RB contributed to editing this paper. MØ contributed to experimental design, data interpretation and editing this paper. DB contributed to experimental design, supervised running of all practical aspects of the trial, and supported data analysis and interpretation, and editing of this paper.

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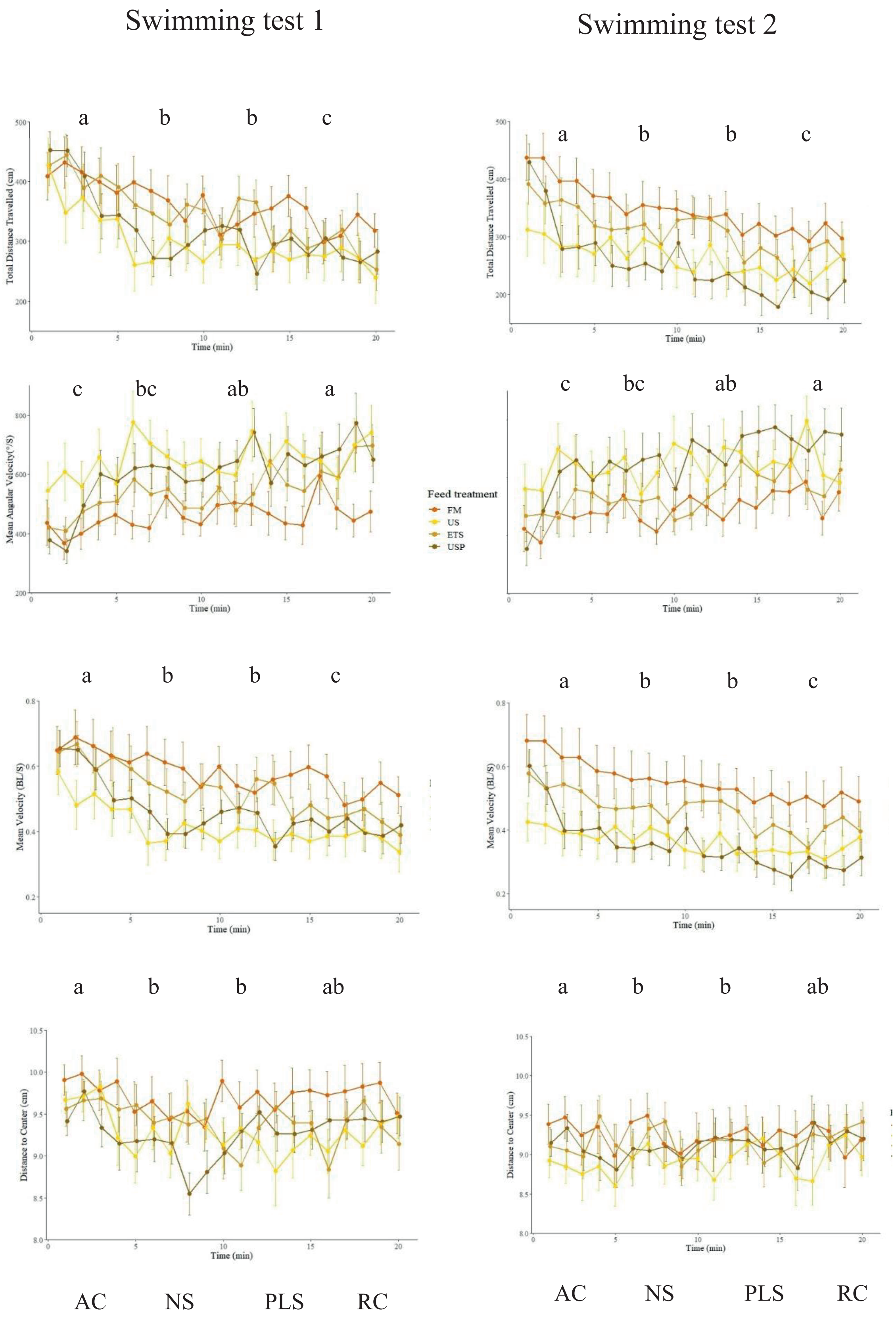
1 Data Availability Statement

The data for gut microbiome will be made available as a BioProject on the Sequence Read Archive when this manuscript is formerly submitted.

Paper I

Supplementary material


Swimming test 1
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Feed treatment
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Distance to Center (cm)
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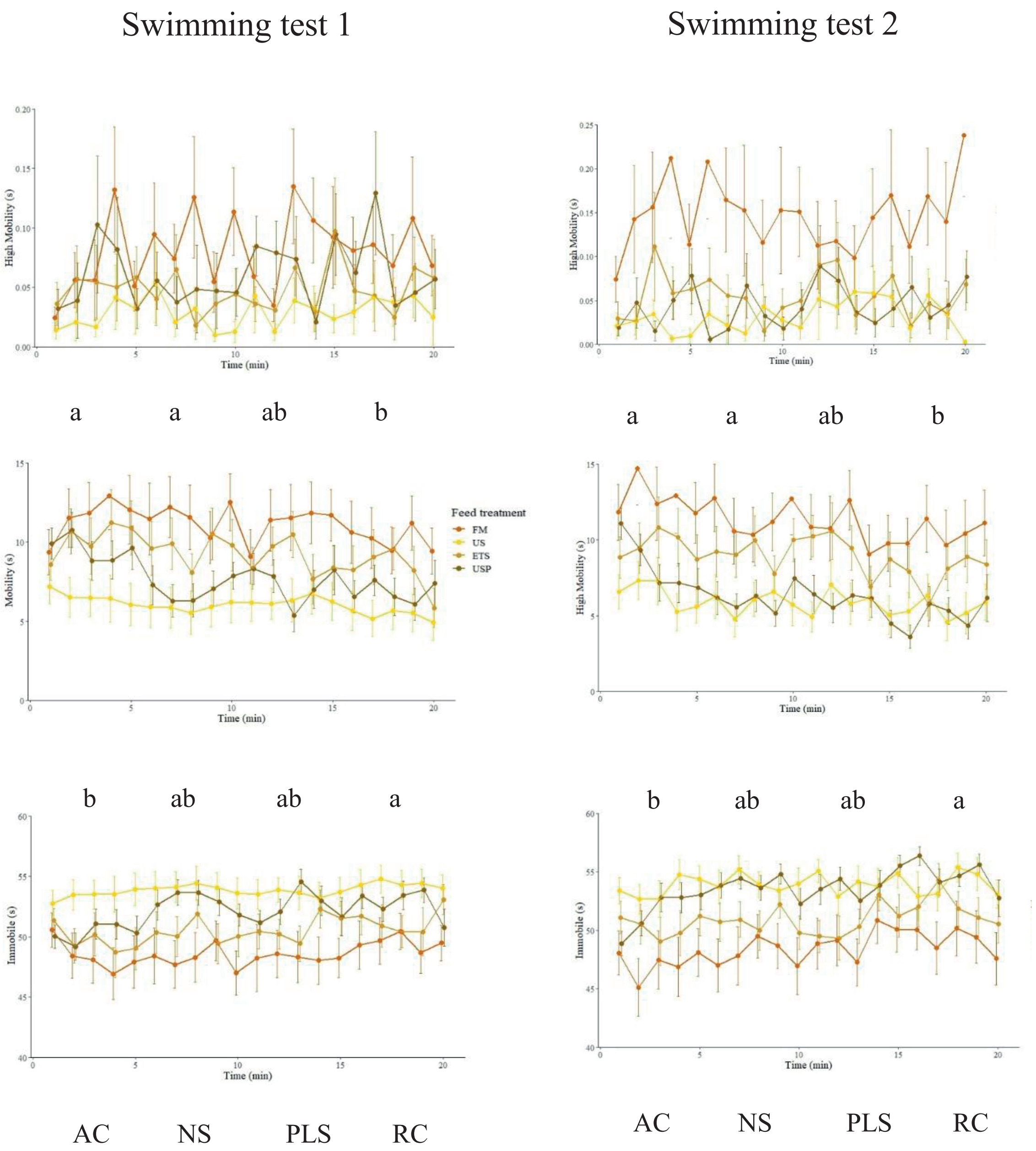

Figure S.1. The swimming activity of Atlantic salmon fed different experimental diets during swimming test 1 and swimming test 2. Fish were filmed in individual arenas for a total of 20 minutes. Each swimming test was split into four virtual periods. AC, Acclimation period 0-5 minutes. NS, normal swimming period 5-10 minutes, after which the bottom-light of the arena was switched off and then on again after 3 seconds. PLS, post light stressor period 10-15minutes. RC, recovery period 15-20minutes. Lower case letters represent significantly different mean value between periods different colours indicate the feed treatment.

Paper II

Feeding Black Soldier Fly Larvae (*Hermetia illucens*) reared on food waste alters gut characteristics of Atlantic salmon (*Salmo salar*).

Black Soldier Fly larvae alter Atlantic salmon gut characteristics

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Abstract

The Atlantic salmon (*Salmo salar*) aquaculture industry is growing, and with it, the need to source and optimise sustainable ingredients for aquafeeds. Black soldier fly (BSF) larvae (*Hermetia illucens*) have received increasing research attention since they are a good source of protein that can efficiently convert a wide range of low-value organic material into valuable resources. This study investigated the impact of three differently processed BSF meals, an untreated BSF diet (BSFC+), a dechitinated BSF diet (BSFC-) and a fermented BSF diet (BSFC+P+) at a 10% inclusion level replacing fish meal in a fish meal control (FM) and a marine and soy protein concentrate based control diet (SPC). Growth performance, gut microbiome and gut histology of salmon fry was assessed. The inclusion and processing methods of BSF showed no adverse impacts on either growth performance or gut histology. However, the gut microbiome of fish was significantly altered by both the protein source and the processing method of the BSF larvae. Fish fed BSFC+, had an increased diversity and evenness of the community compared with conventional protein sources alone, and compared with the other BSF processing methods. However, control diets had a greater presence of lactic acid bacteria and genera associated with faster growing hosts. Fish fed BSF had a high relative abundance of the genus, *Exiguobacterium*, a chitin-degrading bacterium and in BSFC+P+ fed fish this bacterium completely dominated the community, indicating the presence of dysbiosis. Future studies should determine, why *Exiguobacterium* has dominated the community for the BSFC+P+ diet, and if it provides a digestive function to the host and identify bacteria that are indicators of optimal host performance and resilience. The results confirmed that BSF is a promising fish meal replacement for salmon, and it demonstrated that BSFC+ has a potential prebiotic impact on the gut microbiome of Atlantic salmon.

Keywords: microbiome, salmonids, insects, processing, chitin.

1. Introduction

Atlantic salmon (*Salmo salar*) aquaculture has grown rapidly in recent decades in Europe and with it, the demand for high quality feed ingredients (Naylor et al., 2009). Salmon are carnivorous and require high levels of dietary protein, particularly during early developmental phases (NRC, 2011). Traditionally, salmonid feeds relied heavily on fish meal, but as demand has grown and exerted increasing pressure on already exploited wild capture fisheries, sustainable and scalable alternatives were needed by the industry (Tacon et al., 2011; Tacon and Metian, 2009; Ytrestøyl et al., 2015). In recent decades soy protein concentrate has replaced a large proportion of the fish meal in salmonid diets, yet challenges and bottlenecks remain for the use of plant ingredients (Aas et al., 2019). Hence, there is a need for alternative sources of protein for Atlantic salmon feeds that can reduce the pressure on marine protein sources and which simultaneously complements plant protein meals in commercial formulations (Turchini et al., 2019).

Insect meals are a promising alternative in aquafeeds and as a fish meal or plant protein replacement (Barroso et al., 2014; Henry et al., 2015; Weththasinghe et al., 2021b). They have a suitable nutritional profile and are part of the natural diet of many fish species (Lock et al., 2018). They can be produced in high density with low water use and methane emissions, and production is not location dependent (Rumpold and Schlüter, 2013; Van Huis and Dunkel, 2017). Additionally, insect meals can be reared on a wide range of organic substrates, efficiently converting low-value biomasses such as vegetable food waste, to valuable proteins for use in feed applications (Ojha et al., 2020). This provides benefits for the development of a circular economy which reduces the impact of feed and food production and as such is prioritised in European legislation (EC Directive No. 2008/98) (Barragan-Fonseca et al., 2017; Zarantoniello et al., 2020). Insect meals derived from black soldier fly (BSF) larvae (*Hermetia illucens*) have received increasing attention in the salmon aquafeed literature (Askarian et al., 2012; Belghit et al., 2018; Fisher et al., 2020; Weththasinghe et al., 2021b, 2021a). The reduced production cost has led to increased interest and availability of BSF for feed producers (English et al., 2021; Henry et al., 2015; Tomberlin and van Huis, 2020). Existing research indicates that BSF is a promising alternative to fish meal without adverse effect on the physio-chemical qualities of the fillet (Bruni et al., 2020). Yet, the processing of BSF for use in aquafeeds is not fully optimised, and there is thus a call to optimise the application of this novel protein source to maximise growth performance, as well as health and welfare of fish (Henry et al., 2015).

Both the type of protein (Gajardo et al., 2017), and processing methods can alter the gut microbiome of Atlantic salmon (Catalán et al., 2018). During early developmental stages of salmonids the gut microbiome is particularly malleable and strongly influenced by the diet (Michl et al., 2019, 2017) and during the first-feeding period, intestinal microbiomes are established (Dehler et al., 2017). The gut microbial community of fish has important consequences for health resilience, disease resistance (López Nadal et al., 2020; Yukgehnaish et al., 2020), growth performance (Perry et al., 2020), metabolism (Dvergedal et al., 2020), digestion and nutrient uptake (Ghanbari et al., 2015; Llewellyn et al., 2014). The existing research indicates that the presence of certain bacteria such as lactic acid bacteria (LAB) can have functional benefits for digestive function and immune development of the fish, which are highly desirable for commercial aquaculture (Ringø et al., 2018). Few studies exist on the impact of insect meals on the gut microbiome of salmonids, but existing research demonstrates feeding BSF larvae to juvenile Rainbow trout (*Oncorhynchus mykiss*) increased both alpha diversity and presence of LABs in the distal gut (Huyben et al., 2019) and increased *Firmicutes* in the digesta (Bruni et al., 2018a). In Atlantic salmon, BSF altered the gut digesta microbial community and increased richness and diversity during the seawater phase (Li et al., 2021). The impact of BSF on the gut microbiome in the early feeding stages of Atlantic salmon during the freshwater stage is lacking from the existing literature.

Novel processing techniques provide a promising strategy for optimised BSF meal inclusion in aquafeed which is currently limited to only partial inclusion, thus, further research is needed to improve its nutritive value (Barragan-Fonseca et al., 2017; Kroeckel et al., 2012). Fermented feed ingredients have improved application in aquaculture feeds with positive effect on nutritional quality and promoting health and resilience in farmed fish (Catalán et al., 2018; Dawood and Koshio, 2020; Ringø et al., 2020). Fermentation of insects with LABs could lengthen their shelf-life, thus increasing their microbial safety (Klunder et al., 2012). Fermenting feed ingredients can enhance their nutritional value for aquaculture by improving digestibility (Refstie et al., 2005) and driving potentially beneficial changes to the gut microbiome (Catalán et al., 2018). Probiotic LABs have been added to BSF to enhance diets for freshwater crayfish (*Chera cainii*) (Foysal et al., 2021) but the impact of bacterial fermentation of BSF for Atlantic salmon fry diets has yet to be investigated. A limitation to the use of insects such as BSF in aquafeeds is the chitin content in their exoskeleton, which is a highly abundant mucopolysaccharide polymer of N-acetyl-d-glucosamine, (1–4)-linked 2-acetamido-2-deoxy-β-d-glucan (Henry et al., 2015; Li et al., 2019; Park and Kim, 2010; Zarantoniello et al., 2020). Chitin has been considered a potentially problematic component of BSF and has been suggested to cause reduced feed utilisation in Atlantic salmon (Olsen et al., 2006) and that dechitination could be beneficial (Weththasinghe et al., 2021a). Contrary to this, other research indicates that chitin may have beneficial prebiotic effects for fish (Ringø et al., 2006), including increased alpha diversity in Rainbow trout gut microbiomes (Huyben et al., 2019), and altered the gut microbiome in Atlantic salmon (Askarian et al., 2012). There is a need to determine the impact of chitin on the gut bacteria of farmed fish (Zhou et al., 2013) and to use molecular methods to elucidate the impact to community composition and complement existing research for Atlantic salmon (Askarian et al., 2012).

Replacing conventional proteins with BSF in salmon diets may reduce the presence of gut inflammation (Weththasinghe et al., 2021b) which can have important consequences for health, welfare and nutrient uptake (Refstie et al., 2000). This impact could be particularly important at very early developmental stages when the gut morphology is developing during and following first feeding (Sahlmann et al., 2015). Existing studies indicate that BSF does not negatively alter Atlantic salmon gut health during the seawater phase even when all fish meal in the diet is substituted (Li et al., 2020) or in the freshwater phase when 85% of protein was from BSF (Li et al., 2019). In Rainbow trout, it has been indicated that the presence of BSF may even decrease inflammation of the gut associated with soybean meal intestinal enteritis (Kumar et al., 2021). It will be important to understand how different processing methods effect juvenile Atlantic salmon gut histology to determine the optimal method and most suitable for combination with conventional aquafeed proteins.

The objective of this study was to optimise the use of BSF sourced from a circular bioeconomy model for salmonid aquafeeds. The impact of three differently processed BSF meals, an untreated BSF diet (BSFC+), a dechitinated BSF diet (BSFC-) and a fermented BSF diet (BSFC+P+) on growth performance, gut microbiome and gut histology of Atlantic salmon fry was assessed. This study answers a call in the literature to further elucidate the impact of insect meals for farmed Atlantic salmon (Lock et al., 2018).

2. Materials and Methods

2.1. *Experimental animals and study design*

Atlantic salmon (*Salmo salar*) hatched by Stofnfiskur Ltd. (Vogar, Iceland) and reared at 5.5°C, was brought to first feeding using standard commercial techniques and commercial start-feed diet BioMar Inicio-plus (Grangemouth, United Kingdom) of 0.5mm pellet size and at a water temperature of 10°C. Fry were transferred to Matís Aquaculture Research Station (MARS) on March 1st 2018, where they were acclimated for one week to the study facilities. All fish within the experiment were individually weighed following a 12-hour fasting period under anaesthetic (2-phenoxyethanol of 300ppm). Fish were split into twenty 20L-White circular PVC tanks, in quadruplicate for each feed treatment. Each tank contained 30 individual fish with similar initial weight (1.34±0.2g). Fish were kept at 9.0±0.5°C under 24-hour photoperiod of 40±10 lux, oxygen levels were maintained above 80% saturation. Fish were fed with the experimental feed treatments for 75 days. The experiment was performed following European and Icelandic guidelines and within the permits and licenses of the MARS facility.

2.2. *Production and processing of insects*

Insect biomass was sourced from existing black soldier fly producers Better Origin (Cambridge, United Kingdom). Larvae were reared at 28°C at 70% relative humidity in 80x90cm trays. Larvae were fed on organic substrate consisting of 80% shredded potatoes and 20% spent brewer´s yeast, sourced from Milton Brewery (Cambridgeshire, UK). Larvae were harvested at the 5th and 6th instar stages and were euthanised by exposure to cold. Larvae were washed in cold water to remove residual detritus and water-soluble dirt. The biomass was blanched to partially pasteurise the material. Three different treatments of the biomass were applied to produce three types of insect meal. An untreated black soldier fly meal (BSFC+) was sterilised by autoclaving (121°C for 15 minutes). A chitin-negative black soldier fly meal (BSFC-) was processed with a proprietary dechitination apparatus which separated the solid chitin by-product fraction and a liquid protein and fat-rich product fraction. The liquid fraction was then sterilised (121°C for 15 minutes). A processed black soldier fly meal (BSFC+P+) was sterilised and then subjected to bioprocessing (fermentation with *Pediococcus acidlactici* at 37°C). All insect meals were then dried

(80°C for 24 hours) and milled to reduce the particle size for feed inclusion (IPHARMACHINE, Germany). The nutritional profile of each insect meal is provided in (Table 1).

2.3. *Experimental feeds and feeding*

There were five dietary treatments formulated for this investigation. A fish meal-based control (FM) diet and a commercially comparable control with marine and soy protein concentrate based control (SPC) and three insect meal diets, BSFC+, BSFC-, BSFC+P+ Insect meal was included at 10% in the feed formulation, primarily replacing fish meal and fish oil (Table 2). The nutritional composition of all protein sources used are included in Table 1. The diets were produced through cold pelletisation and drying followed by a crumbling process (<0.5mm diameter crumbs) at Matís ohf., Iceland. All dry ingredients were milled to bring all materials to equal particle size (IPHARMACHINE, Germany). Dry ingredients were then homogenised in a standard food mixer (KitchenAid, USA) and the mix was returned to the same mill to improve the homogeneity of the feed. The dry mix was returned to the food mixer and fish oil was added while simultaneously mixing. A small volume of water was added to produce the ideal consistency for the next stage (500ml) The mix was then spread thinly onto a drying tray lined and oven dried at 30°C (Convotherm, Eglfing, Germany) until moisture content was <10%. The dried material was then crumbled to 0.5mm diameter crumbles. During the 75-day feeding trial, tanks were fed 15 times per day by electric belt-feeder between the hours of 09:00 and 01:00. All tanks were fed identical volumes, with 15% excess based on feed requirements at this developmental stage.

Table 1. Chemical composition of the protein sources used.

	Protein Raw Materials					
Composition (*g/kg*)	Fish meal	Soy protein concentrate	Corn gluten meal	BSFC+	BSFC-	BSFC+P+
Dry Matter	909	925	910	979	916	946
Crude Protein	659	633	582	438	373	437
Crude Lipid	107	2	10	292	308	262
Ash	139	90	23	124	101	114
Essential Amino Acids (*g/kg*)						
Arginine	42.3	42.9	18.6	24.4	21.2	21.5
Histidine	18.2	15.5	12.2	14.7	11.6	13.8
Isoleucine	30.2	27.5	23.2	19.8	16.8	19.3
Leucine	57.1	47.4	96.1	32.7	26.6	32.1
Lysine	58.8	37.0	9.1	31.0	28.6	31.0
Methionine	19.6	9.0	14.3	8.2	8.3	8.2
Phenylalanine	28.9	32.1	37.2	21.3	19.3	21.2
Threonine	33.3	25.1	20.3	19.8	16.9	19.8
Valine	37.8	28.5	28.0	28.3	20.0	28.0
Tryptophan	7.6	8.6	3.1	7.3	6.4	7.5
Non-Essential Amino Acids (*g/kg*)						
Alanine	47.2	26.3	52.7	29.1	20.7	29.7
Aspartic acid	69.2	71.3	36.2	47.3	42.3	46.0
Glycine	50.3	25.6	17.4	27.0	19.0	27.1
Glutamic acid	105.0	114.0	130.0	58.4	50.7	53.9
Cystein + Cysteine	5.8	8.6	11.4	3.5	3.5	3.5
Tyrosine	24.6	22.3	30.6	34.3	26.7	32.6
Proline	32.1	30.9	55.7	24.1	17.2	23.8
Serine	32.6	32.4	33.5	20.0	15.3	22.2

Table 2. Feed formulation and chemical composition for used.

	FM	SPC	BSFC+	BSFC-	BSFC+P+
Formulation (g/kg)					
Fish Meal [a]	630.0	467.6	405.9	417.0	406.8
Pre-gelatinised Wheat [b]	180.8	133.1	117.0	108.6	113.1
Vitamin-Mineral Premix [c]	10.0	10.0	10.0	10.0	10.0
Fish Oil [a]	101.2	119.7	97.4	94.7	100.5
Soy Protein Concentrate [a]	0.0	191.6	191.6	191.6	191.6
Corn Gluten Meal [a]	78.0	78.0	78.0	78.0	78.0
BSFC+	0.0	0.0	100.0	0.0	0.0
BSFC-	0.0	0.0	0.0	100.0	0.0
BSFC+P+	0.0	0.0	0.0	0.0	100.0
Composition (g/kg)					
Dry Matter	867	884	909	922	894
Crude Protein	467	484	492	503	495
Crude Lipid	219	202	199	202	217
Ash	75	88	96	97	96

[a] Laxá hf. Krossanes, Iceland

[b] Emmelev A/S, Denmark

[c] Laxa salmon premix 2006, Trouw Nutrition, The Netherlands

2.4. *Growth Performance*

After 75 days of feeding, all individual fish from each treatment and replicate tanks were weighed (wet weight (g)) and measured (total length (cm)), following a 12-hour fast. From this data the Fulton´s Condition Factor (K) and Specific Growth Rate (%) (SGR) over the study period could be calculated: K = (Weight/Total Length^3) × 100 and SGR = ((Ln(Final Weight)-Ln(Initial Weight)) × 100)/*t*, where *t* is the number of days over which the trial was run. Mortality was monitored daily throughout the feeding trial.

2.5. *Gut Sampling*

After the assessment of growth performance, all fish were left for one week to recover from handling and fed the same experimental diets. At the end of this recovery period, samples were taken for gut microbiome analysis and gut histology analysis. For both sample types, fish were fed just two hours prior to sampling to ensure high volumes of gut content. For the gut microbiome analysis, twelve fish per feed treatment three fish per tank were randomly selected. Selected fish were euthanised with a lethal dose of anaesthetic (phenoxyethanol) 600ppm and the outside of the fish washed in 90% ethanol followed by sterile distilled water. The digesta from the distal part of the gastro-intestinal tract was extracted under sterile conditions. Triplicate samples of each dietary treatment were collected. All samples were stored at -80°C prior to downstream processing. A further twelve fish per feed treatment (three fish per tank) were randomly selected for gut histology sampling. Selected fish were euthanised with a lethal dose of anaesthetic (phenoxyethanol) 600ppm. Fish were dissected to remove the distal gut, and gut content was washed out of the gut section with phosphate buffered solution (PBS). Samples were stored in 10% buffered formalin for 48-hours and then transferred to 70% ethanol and stored at 4°C prior to downstream processing.

2.6. *DNA extraction, PCR amplification and sequencing*

Distal gut digesta samples and dietary treatment samples for gut microbiome analysis were transferred to a sterile 2ml Eppendorf tube with 300µl of sterile 1mm diameter sterile silica beads (BioSpec Products, United States). 800µl of CD1 from the QIAamp PowerFecal Pro DNA kit (QIAGEN, Germany) was added to the Eppendorf tube. Samples were vortexed for 5 seconds and shaken at maximum speed (30Hz) in a laboratory mixer mill (Retsch MM400) for 1 minute. The supernatant (~800µl) was transferred to the PowerBead Pro Tube from the QIAGEN QIAamp PowerFecal Pro DNA kit. The protocol for this DNA extraction kit was then followed according to manufacturer instructions, and finally eluted with 80µl of solution C6. An negative control with no material was also run to ensure no contamination occurred during the DNA extraction protocol. DNA concentrations were measured in 2µl of sample using the Invitrogen Qubit dsDNA BR Assay kit (Invitrogen, Carlsbad, CA, USA). DNA were diluted to 4ng/µl in a 50µl aliquot. Samples were then subjected to PCR of a region covering the V3-V4 regions of the 16S rRNA gene with a universal bacterial primer pair S-D-Bact-0341-b-S-17 (5′−CCTACGGGNGGCWGCAG-3′)/S-D-Bact-0785-a-A-21(5′-GACT- ACHVGGGTATCTAATCC-3′) (Klindworth et al., 2013). The PCR master mix included the diluted DNA, nuclease-free water, Q5 High-Fidelity DNA polymerase (New England Biolabs, Ipswich, USA), Q5 GC Enhancer, 0.5µM of each primer containing Illumina overhang adapters, and 1x Q5 Reaction buffer, 200µM dNTPs (New England Biolabs, Ipswich, USA). Included in the PCR were both positive and negative samples to monitor for successful amplification and absence of contamination of the target region only. The thermocycling protocol had an initial denaturation step (98°C for 30s), followed by 35 cycles of, denaturation (98°C for 10s), annealing (52°C for 30s), and extension step (72°C for 30s), with a final extension (72°C for 2min). Libraries were multiplexed with Nextera XT v2 barcodes (Illumina, USA), normalised using *Sequel-Prep* Normalisation Plates (ThermoFisher Scientific, USA) and sequenced on a MiSeq desktop sequencer (Illumina, USA) using v3 chemistry and 2 × 300 cycles.

2.7. *Histological sample processing and analysis*

Distal gut tissues were equilibrated in xylene and embedded in paraffin wax following standard histological procedures. Sections of 4µm thickness were cut and stained with haematoxylin and eosin (HE). The sections were cut longitudinally (perpendicular to the folds). Processing was carried out at the Veterinary department of The Norwegian University of Life Sciences (NMBU). Blind histological examination was performed on images taken using a light microscope with camera connected (Olympus BX51, Tokyo, Japan) with a magnification of (x100). Tissue morphology was evaluated using a semi-quantitative scoring system with scores from 1-3 for lamina propria thickness and submucosal connective tissue width (Barnes et al., 2014; Colburn et al., 2012; Knudsen et al., 2007). The scoring criteria used is described in Table 3.

Table 3. Histological scoring system used on Atlantic salmon fed experiment feed treatments (modified from (Barnes et al., 2014; Colburn et al., 2012; Knudsen et al., 2007)).

Score	Appearance
	Lamina propria of simple folds
1	Thin and delicate core of connective tissue in all simple folds.
2	Lamina propria slightly more distinct and robust in some of the folds.
3	Clear increase in lamina propria in most of the simple folds
	Connective tissue between base of folds and stratum compactum
1	Very thin layer of connective tissue between base of folds and stratum compactum.
2	Slightly increased amount of connective tissue beneath some of the mucosal folds.
3	Clear increase of connective tissue beneath most of the mucosal folds

2.8. *Statistical Methods*

Statistical analyses were performed in R version 3.6.1 (2019-07-05). All tests were two-tailed with a significance level set to $\alpha = 0.05$. To assess growth performance, two dependent variables were statistically assessed, the average tank SGR% and K. A Linear Model (LM) with the package *nmle* (Pinheiro, 2020) was selected where the feed treatment was a fixed factor. To support the statistical robustness of this test and to account for potential variation at the tank level further statistical analysis was run. Firstly, a generalised linear mixed model (GLMM)f with the package *lme4* (Bates et al., 2015) on weight of all fish at the start of the trial, where feed treatment was a fixed factor and tank was a random nested factor of feed treatment to confirm there was no difference across fish body weight between tanks at the start of the trial. Secondly, another GLMM on the weight of all fish at the end of the trial where feed treatment was a fixed factor and tank was a random nested factor of feed treatment.

To assess the microbiome of digesta of the distal gut and the dietary treatments, demultiplexed FASTQ files from Illumina were processed to produce amplicon sequence variants (ASVs) using the DADA2 package version 1.16.0 (Callahan et al., 2016) in Rstudio version 4.0.2(Team, 2020). The function filterAndTrim set variables as, truncLen=c(280,250), trimLeft= 21, maxN=0, maxEE=c(2,2), truncQ=2. The SILVA database version 138 was used to assignTaxonomy to the ASVs (Quast et al., 2013). The microbial community was analysed using R packages phyloseq (McMurdie and Holmes, 2013), microbiome (Lahti and Shetty, 2017) and vegan (Oksanen et al., 2020), and visualised with ggplot2(Wickham., 2016). The average number of reads per sample output from the DADA2 pipeline were 16213 ± 7515 for all samples except for a single digesta sample from the SPC diet tank 3 that did not produce reads and was removed from downstream processing. Three PCR negative samples were also sequenced to control for any contamination during the sample processing steps, and were used as controls to remove suspected contamination from the samples using the package *decontam* (Davis et al., 2017). The prevalence method and a threshold of 0.5. For all subsequent analysis, the read depth was normalised across samples with the function *rarefy_even_depth* to the sample with the lowest read depth. Raw 16S rRNA gene amplicon reads are deposited in the Sequence Read Archive under BioProject ID PRJNA733893 available at http://www.ncbi.nlm.nih.gov/bioproject/733893. The gut microbial community of fish fed the study diets, and the microbial community of the diets were quantitatively analysed using alpha and beta diversity measures. The selected alpha diversity measures where the observed richness of ASVs, Shannon diversity, Chao1 diversity and Pielou´s Evenness. For the gut samples, a GLMM was used to assess if there was a significant difference in these alpha diversity measures between the fish gut digesta fed different dietary treatments. In this model feed treatment was a fixed factor and tank was a nested random factor of feed treatment. The random nested factor of tank was tested by a likelihood ratio test (LRT) (Fox et al., 2011). For the diet microbiome, a linear model (LM) was selected to assess if the alpha diversity measures between the diet samples were significantly different, in this model the feed treatment was a fixed factor. Post-hoc testing was carried out using a Tukey test. The gut microbiome community assemblage for fish fed each of the dietary treatment and the microbiome community of the diets respectively were transformed using a *Bray-Curtis* dissimilarity matrix and non-metric multidimensional scaling was applied. An Analysis of similarity (ANOSIM) test was conducted to assess for significant difference between and within fish fed different feed treatments. To further investigate the microbiome community assemblage the relative abundance of taxa as a proportion was visualised at the phylum level in stacked bar plots for direct comparison. The genus level was then visualised, with all genera present at less than 1% of the relative abundance grouped into a category called “Other” and the genera present at greater than 1% relative abundance were visually displayed using boxplots for each feed treatment. To assess the gut histology, a non-parametric Kruskal-Wallis test was run on the two gut histology variables, lamina propria of simple folds, and connective tissue at base of folds. In this model, feed treatment was a fixed factor.

3. Results

3.1. *Growth performance*

There was no significant difference in SGR% among the dietary treatments with similar average values (Figure1A). There was also no significant difference in K between feed treatments with similar average values (Figure 1B). There was no significant difference in fish weight between dietary treatments or between tanks at either the start or the trial or the end of the trial.

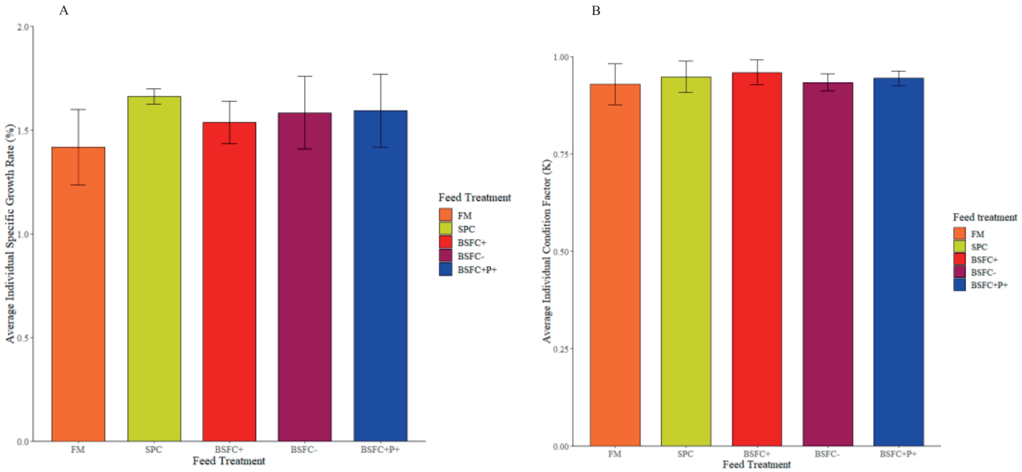

Figure. 1. Bar plot of **(A)** the average individual Specific Growth Rate (SGR) (%) and **(B)** the average individual Condition Factor (K) of fish fed the experimental feed treatments. Bars represent the standard deviation of the data.

3.2. *Gut microbiome*

There was a significant difference in all alpha diversity measures among dietary treatments. The observed richness of ASVs (Figure 2A) was highest in the BSFC+ diet, followed by BFC- and SPC, which all had higher values than the FM control diet, and BSFC+P+ had the lowest observed richness of all the dietary treatments tested. The Shannon diversity index (Figure 2B) was higher is BSFC+ than all other treatments expect for SPC, BSFC+P+ had the lowest values compared to all other dietary treatments and FM had the second lowest values. The Chao1 diversity (Figure 2C) followed an identical pattern to the Shannon diversity index, and BSFC- showed a larger range of diversity values. Pielou´s evenness (Figure 2D) was significantly higher in BFC+ and SPC, followed by FM and BSFC-. The lowest community evenness was found in BSFC+P+. The alpha diversity indices in the diet samples (Figure S1) differ slightly from the digesta. For the observed richness of ASVs (Figure S1 (A)), and the Chao1 diversity (Figure S1 (C)), BSFC- diet values are closer to the BSFC+ values than for the digesta. Whereas, for the Shannon diversity (Figure S1(B)), and Pielou´s evenness (Figure S1(D)), BSFC- had the lowest values, and BSFC+P+ values for Pielou´s evenness was closer to BSFC+ and the FM, and SPC diet samples.

The gut microbial composition of the fish fed different diets was significantly different. There was larger difference in the microbial communities of fish fed different diets, than in fish within the same dietary treatment group (ANOSIM P = 0.001, R0.8949) the clustering of the NMDS (stress = 0.14) (Figure 3) is quite distinct. This suggests the dietary treatments in this study created fish with distinct gut microbial communities in the digesta of the distal gut. The FM and SPC control diet clusters were close together, and that the BSFC+ and BSFC- diet clusters were close together, but BSFC+P+ was clustered away from all other dietary treatments. At the phylum taxonomic level (Figure 4), the gut communities were dominated by *Firmicutes* in all dietary treatments (FM=0.55±0.31, SPC=0.87±0.15, BSFC+=0.76±0.15, BSFC-=0.79±0.09, BSFC+P+=0.97±0.03). The second most dominant phyla were *Actinobacteriota* (FM=0.16±0.26, SPC=0.05±0.06, BSFC+=0.12±0.04, BSFC-=0.15±0.07, BSFC+P+=0.03±0.02) and *Proteobacteria* (FM=0.27±0.32, SPC=0.07±0.08, BSFC+=0.12±0.12, BSFC-=0.06±0.07, BSFC+P+=0.00±0.00). For BSFC+P+ fed fish it *Proteobacteria* was absent. There was also some tank effect present, which was most noticeable for fish in tank 4 fed the FM control which had much higher abundance of *Proteobacteria* than any other fish within that treatment. The phyla present in the diet microbiota samples (Figure S2) follow similar trends to the phyla in the digesta, with a few exceptions, across all diet samples. There was a greater relative abundance of *Actinobacteriota,* and the diet microbiota sample for BSFC+P+ has a greater proportion of *Proteobacteria* than in the fish digesta. For the BSFC- diet samples, there was a greater presence of *Bacteroidota* compared to the fish digesta samples.

At the genus taxonomic level (Figure 5) there were seven genera that with relative abundance above 1% of the community assemblage. All other genera present showed a low abundance. For the genus *Bacillus* there were high levels in both fish fed the BSFC+ and BSFC- diets, low levels in those fed the SPC diets, but none present in either FM or BSFC+P+ diets. For *Clostridium_sensu_stricto_1*, *Enterococcus, Lactobacillus* and *Peptostreptococcus*, the same pattern was observed, with moderate levels for the FM control, the highest levels for SPC control, and decreasing levels for the BSFC+ and BSFC- diets and none present for the BSFC+P+ diet. A similar pattern was observed for *Clostridium_sensu_stricto_7,* except none were present in the FM control. The only genera present above 1% in BSFC+P+ was *Exiguobacterium*, which was detected in high relative abundance for the BSFC- diet, with low levels for the BSFC+ diet and none present in either FM or SPC control diets. The genera detected in the diet microbiota samples (Figure S3) have several bacteria present at greater than 1% relative abundance that were not detected at greater than 1% in the digesta. The diet samples for BSFC+P+ were more diverse than for the resulting digesta samples. There were no LABs greater than 1% relative abundance in the feeds. The relative abundance of the genera, *Clostridium_sensu_stricto_1* and *Clostridium_sensu_stricto_7* was higher in the FM feeds than in the resulting FM digesta.

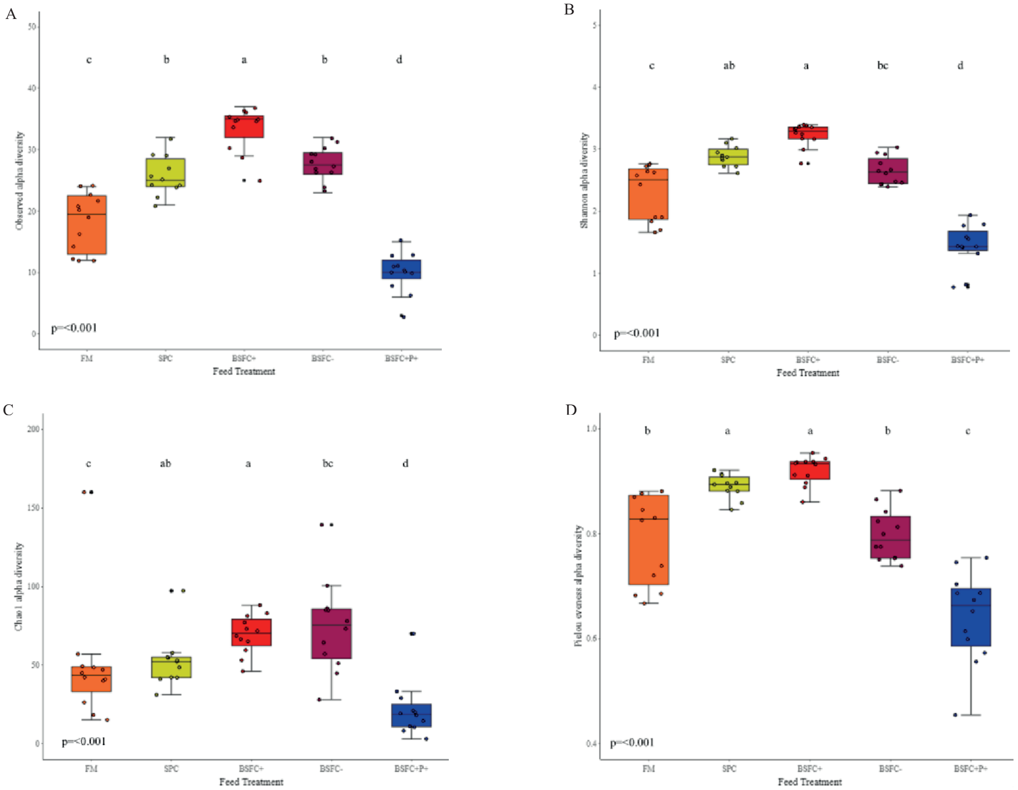

Figure. 2. Box plots of alpha diversity measures for the gut digest of fish fed each dietary treatment **A)** The observed richness of ASVs, **B)** Shannon diversity, **C)** Chao1 diversity, **D)** Pielou´s evenness. The P value for each variable is displayed on its respective graph.

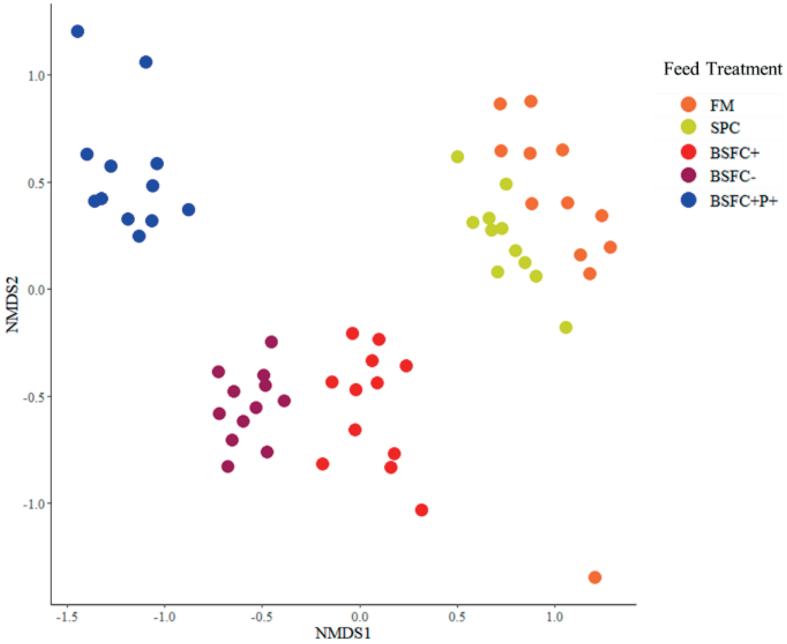

Figure. 3. Non-metric multidimension scaling (NMDS) of study fish from each dietary treatment. Each point represents a single fish and colour indicates the feed treatment inclusion level.

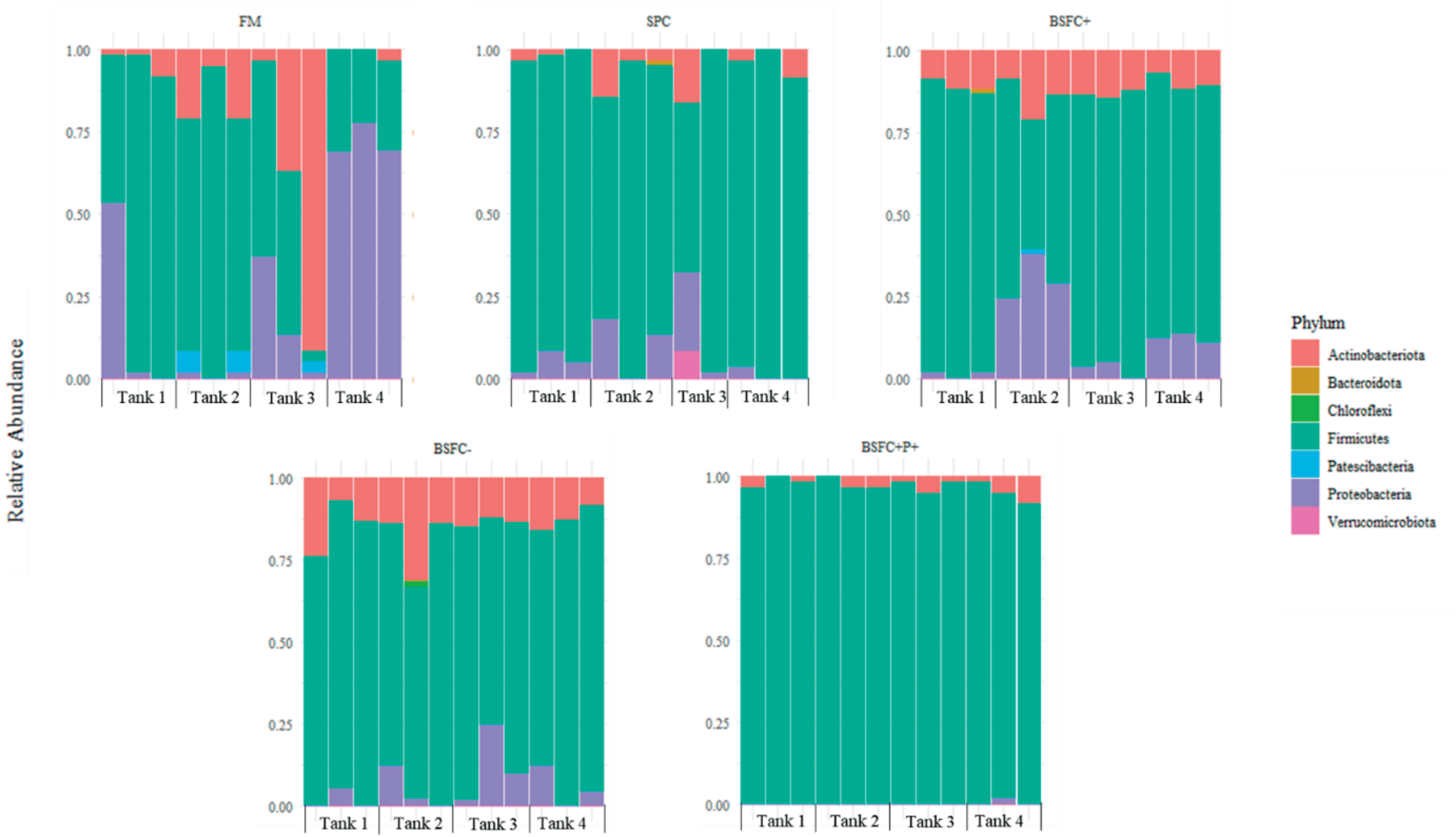

Figure. 4. Stacked bar plot of gut bacterial composition using relative abundance of the most common phyla (above 1% relative abundance) for the study fish from each dietary treatment.

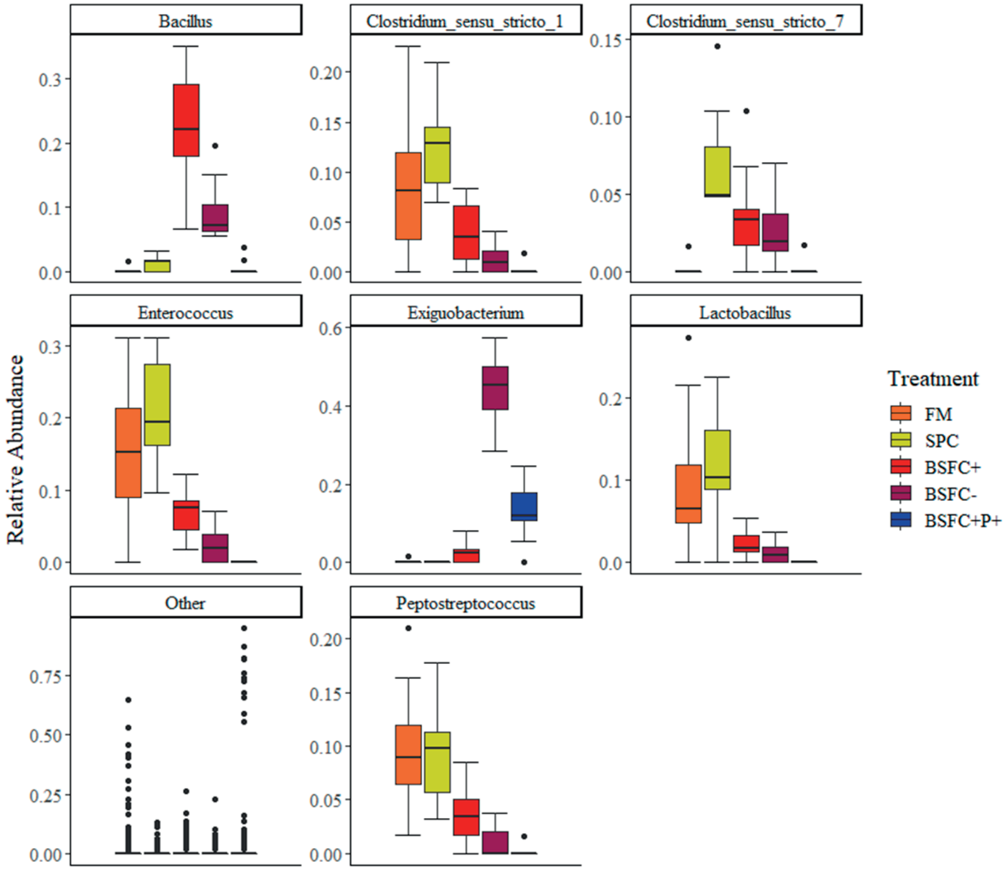

Figure. 5. Boxplots of gut bacterial composition using relative abundance of the most common genera found in fish from each dietary treatment.

3.3. *Gut histology*

There was no significant difference in lamina propria of simple folds between the dietary treatments with similar average values observed (FM: 1.18±0.40, SPC: 1.25±0.45, BSFC+: 1.18±0.40, BSFC-: 1.33±0.49, BSFC+P+:1.17±0.39). There was also no significant difference in connective tissue at base of folds between the dietary treatments with similar average values observed (FM: 1.00±0.00, SPC: 1.00±0.00, BSFC+: 1.00±0.00, BSFC-: 1.08±0.29, BSFC+P+:1.08±0.29).

4. Discussion

The present study reports the impact of BSF reared on food waste on the growth performance, gut microbiome, and gut histology of Atlantic salmon fry. Three different processing treatments of BSF (an untreated BSF diet (BSFC+), a dechitinated BSF diet (BSFC-) and a fermented BSF diet (BSFC+P+)) were compared to a fish meal control diet (FM) and a mixed fish meal and soy protein concentrate control diet (SPC) to optimise the application of BSF in formulated aquafeeds.

Growth performance of Atlantic salmon fry in this study was comparable across all dietary treatments for both the controls and the 10% inclusion of BSF, and all values were within normal range for the freshwater (FW) phase and experiment temperature (Nathanailides et al., 1995; Ørnsrud et al., 2002). The type of processing applied to the BSF did not have any measurable impact on either SGR (%) or K. Existing studies reported similar findings for low to moderate protein substitution levels of BSF, where adding up to 12.5% BSF meal did not adversely affect growth performance for FW phase Atlantic salmon (Weththasinghe et al., 2021a). The same study reported that higher substitution levels of 25% reduced growth performance. Whereas, another study on Atlantic salmon fry showed no adverse impacts on growth performance with inclusion up to 20%, but reduced performance at 30% inclusion (Fisher et al., 2020). A further study, showed that 85% of protein replacement with BSF had no adverse impact on growth performance (Belghit et al., 2018). In post-smolt Atlantic salmon, 100% replacement of fish meal has been reported without compromising growth performance (Belghit et al., 2019; Lock et al., 2016). The results of the present study further confirm the existing findings, that lower inclusion levels of BSF provide comparable SGR and K to conventional protein sources for Atlantic salmon during the FW phase. At this low inclusion level of 10%, the removal of chitin from BSF did not produce marked benefit to growth performance, suggesting that chitin is not a limiting factor when low levels of BSF are used for aquafeed applications as some studies have suggested (Kroeckel et al., 2012; Olsen et al., 2006; Weththasinghe et al., 2021a; Xiao et al., 2018). This study suggests that fermentation of insect proteins did not provide any measurable benefits to growth performance in juvenile salmonid diets. There is lack of existing research that assesses the impact of fermenting insect protein sources for aquafeeds. Existing studies have observed improved growth performance in several carp species (*Catla catla*, *Cirrhinus mrigala, Labeo rohita, Hypophthalmychtyhys molitrix*) (Rangacharyulu et al., 2003) and broiler chickens (Rao et al., 2011) fed fermented silkworm pupae silage. Weight gains have been observed for broiler chickens fed up to 25% of fermented sago larvae replacing fish meal (Sjofjan and Adli, 2021). Future studies should elucidate the impact of processing to improve protein quality of BSF in salmonid diets. It would also be valuable to assess if dechitination has an impact on growth performance when BSF is fed at higher inclusion levels, since the upper limits of BSF in the diet of Atlantic salmon are unclear (English et al., 2021), and dechitination may influence maximum inclusion levels. It will be important to optimise all aspects of BSF production to account for discrepancies between existing findings which may be driven by several factors, such as differences in rearing conditions (English et al., 2021), variable substrates used during BSF production (Zarantoniello et al., 2020), and different processing of the BSF prior to dietary inclusion (Barragan-Fonseca et al., 2017). Future studies should determine the optimal method to maximise inclusion of BSF as a substitute for conventional aquafeed ingredients (English et al., 2021).

The alpha diversity measures in distal gut digesta of fish fed diets in the present study show that the untreated BSF (BSFC+) had higher diversity, richness, and evenness measures than all the processed BSF dietary treatments and the FM control. BSFC+ also had comparable values as the SPC control diet except for the ASV richness, which was higher in BSFC+. These results indicate that chitin in the BSF may have a prebiotic impact on the gut microbiome of these juvenile fish. This would be beneficial for aquaculture since higher microbial diversity levels and an even community could both be signs of a beneficial gut health and can support productivity (Infante-Villamil et al., 2021). To the knowledge of these authors, the present study is the first to report the impact of BSF on the gut microbiome of juvenile Atlantic salmon during the FW phase. Existing studies in post-smolt Atlantic salmon in the seawater (SW) phase detected similar patterns for alpha diversity as found herein, reporting increased microbial diversity and richness in digesta of fish fed diets with a 15% inclusion of BSF replacing conventional protein sources in a mixed protein source diet (Li et al., 2021). Similarly, in studies with Rainbow trout (*Oncorhynchus mykiss*) fed a diet with 30% BSF larvae and pre-pupae inclusion, replacing fish meal (Huyben et al., 2019) and a 25% and 50% replacement of fish meal with BSF (Bruni et al., 2018a) respectively, alpha diversity increased which was attributed to the presence of chitin (Huyben et al., 2019). Chitin supplementation has also been observed to alter gut microbiota of Atlantic salmon (Askarian et al., 2012) and have a beneficial prebiotic potential for aquafeed inclusion (Bruni et al., 2018a; Ringø et al., 2012). Removal of chitin in the BSFC- diet in the present study reduced the alpha diversity measures of gut microbiome compared with the untreated BSF (BSFC+), diet but the measures were still comparable to those fish fed the SPC diet, except for the community evenness which was lower for BSFC-, but comparable with the FM control fish. This suggests that dechitination does not provide potential benefits for the gut microbiome, and may even have adverse consequences associated with reduced microbial diversity on gut health and resilience (Apper et al., 2016). Fish fed the fermented BSF diet (BSFC+P+) in the present study had the lowest diversity and the most uneven community in their distal gut digesta, indicating possible dysbiosis and limited bacterial competition to protect against pathogens entering the gut, conditions associated with poor health and resilience in fish (Apper et al., 2016; Infante-Villamil et al., 2021). This finding is contrary to indications from the existing literature, that fermenting insects increases the presence of bioactive compounds (Castro-López et al., 2020) and their nutritional value (Kewuyemi et al., 2020) that might be expected to have benefits for the gut microbiome. Further investigation of the impact of insect meal fermentation on the fish gut microbiome will be necessary to determine the causes of the low microbial community diversity and unevenness shown by the present study.

In the present study, the beta diversity of the distal gut digesta microbiota reveals a distinct microbial community composition across dietary treatments. The greatest differences were observed between the community composition of the FM and SPC control diets compared with the BSFC+ and BSFC- diets, with the most distinctly different community composition found in the fermented BSFC+P+ fish. There is strong consensus in the existing literature that the dietary protein source alters the community composition of salmonids (Gajardo et al., 2017; Michl et al., 2017). This has been reported when conventional proteins are replaced with BSF (Li et al., 2021). The dominant phyla in the distal gut digesta of fish across all diet treatments in the present study is comparable to other freshwater stage salmonids, which are commonly dominated by *Firmicutes*, *Proteobacteria* and *Actinobacteria* (Gajardo et al., 2016; Huyben et al., 2019). At the phyla level the strong dominance of *Firmicutes* in the digesta of fish fed the fermented BSF diet (BSFC+P+) explains the unevenness present in this community. At the genus level, the driver of community unevenness and the dominance of *Firmicutes* in fish fed the fermented diet, BSFC+P+ can be identified as the genera *Exiguobacterium*. *Exiguobacterium* is a bacterium that produces chitinase that degrades chitin (Anuradha and Revathi, 2013). In the present study, fish fed any diet containing BSF have *Exiguobacterium* present in this distal gut digesta in an abundance higher than 1%. The same bacteria has been detected in gut microbiome samples from both farmed Atlantic salmon fed commercial feed (Webster et al., 2020), in wild flounder (*Paralichthys adspersus)* (Salas-Leiva et al., 2017) but was not found in the gut microbiome of BSF fed Rainbow trout (*O. mykiss*) using high-throughput sequencing (Huyben et al., 2019). Chitinase activity has immune benefits for fish and presence in the gut microbiome community can be desirable (Zhang et al., 2012). *Exiguobacterium acetylicum S01* isolated from soil was reported to inhibit the growth of fish pathogens and have a desirable probiotic role (Jinendiran et al., 2019a), and as a dietary supplement, improved growth performance and immune responses in goldfish (*Carassius auratus*) (Jinendiran et al., 2019b). Future work should isolate *Exiguobacterium* present in fish species fed BSF and assess its probiotic potential and should confirm that the bacterium is active in the fish gut environment. In the case of the fermented BSF diet in the present study, this bacterium dominates the community, and is driving the observed dysbiosis which may reduce the resilience of these fish and is undesirable (Apper et al., 2016; Lozupone et al., 2012). This suggests that fermented BSF may not be suitable for Atlantic salmon at such as early developmental stage when the gut microbiome is still becoming established (Rodríguez et al., 2015) and this phenomenon requires further investigation. The strong presence of *Exiguobacterium* in fish fed the dechitinated diet (BSFC-) in the present study could be driven by the dechitination process which may have removed bulk chitin but left glycosidic bonds intact that this bacterium was able to efficiently exploit. It will be important for future studies to consider the indirect impacts of BSF processing methods. The present study also observed increased relative abundance of the genus *Bacillus* in both the untreated BSF diet (BSFC+) and the dechitinated BSF diet (BSFC-) compared with both controls. This was also observed in Rainbow Trout (*O. mykiss*) fed BSF diets (Huyben et al., 2019) and in Atlantic salmon fed diets with5% chitin supplementation, where the chitin may have supported the growth of *Bacillus* species by providing additional substrate for growth (Askarian et al., 2012). Two genera of bacteria, *Clostridium_senus_stricto_1* and *Peptostreptococcus* were found in higher relative abundance in the two control diets of the present study and were slightly lower in the untreated BSFC+ diet and even lower in the dechitinated BSFC- diet. These bacteria have been associated with faster growing individuals in Rainbow trout (*O.mykiss*) (Chapagain et al., 2019). No growth benefit was observed in the present study, and it will be important to extend this trial to monitor growth up to harvest size and to determine if the same bacteria are also growth rate indicators in Atlantic salmon. Two lactic acid bacteria (LAB) were detected above 1% relative abundance in fish in the present study, *Enterococcus* and *Lactobacillus*, these were both also in highest abundance in the two control diets, and decreased from BSFC+ to BSFC- diets, to absent in BSFC+P+ fed fish. This is contrary to existing studies that reported an increase in LABs within presence of BSF (Bruni et al., 2018; Huyben et al., 2019). It will be important to determine if this reduced LAB presence has any impact to health and resilience of Atlantic salmon and to establish a cause-effect relationship to elucidate their roles for the host (Gajardo et al., 2017). The microbiota detected in the diet samples appears to influence the resulting microbial community in the fish gut digesta, which has been established in existing studies for salmonids (Gajardo et al., 2017; Li et al., 2021). However, there were notable differences between the microbial communities in both sample types, and the alpha diversity trends and genera present. This is particularly notable for the BSFC+P+ diet microbial community which is not dominated by *Exiguobacterium* in the same manner as the digesta samples for fish fed this diet.

The gut histology of Atlantic salmon fry in the present study did not differ among the dietary treatments and the low scores for histological characteristics suggest there was no adverse impact on gut health and no inflammation was detected for any of the diets fed. The controls of this study were fish meal (FM) and soy protein concentrate (SPC) protein sources which are minimally impactful to gut health in salmonids at the inclusion levels applied (Booman et al., 2018; Krogdahl et al., 2020). This finding confirms existing reports that BSF at low inclusion levels does not negatively impact gut health in salmonids compared with control diets (Elia et al., 2018; Li et al., 2019; Weththasinghe et al., 2021b). One study reported no adverse effect to gut health when BSF replaced 100% of fish meal in Atlantic salmon diets (Li et al., 2020). Furthermore, in a study where soybean meal was fed to Rainbow trout (*O.mykiss*), intestinal enteritis was present and a supplementation of 16% BSF was found to prevent enteritis (Kumar et al., 2021). Future studies should combine BSF with and without chitin with feed ingredients that are linked to poor gut health to investigate possible benefits for Atlantic salmon.

5. Conclusion

Our results support the existing literature that a 10% inclusion of BSF as a replacement for fish meal in formulated feeds, does not adversely impact growth performance or gut histology of Atlantic salmon during the freshwater phase. Furthermore, the BSF processing methods used in this study did not alter the growth performance or gut histology. The gut microbiome of Atlantic salmon in this trial was significantly altered by both the protein source and the processing method of the BSF larvae used. This study revealed that the untreated BSF fed fish, had a potentially prebiotic impact on the gut microbiome community alpha diversity and evenness compared with conventional protein sources alone. Compared with the other processing methods, dechitinated BSF or fermented BSF. Both control diets had a greater presence of both LABs and potential bacterial indicators of faster growing salmonid host than any of the diets containing BSF. All fish fed a diet containing BSF had a high relative abundance of the genus, *Exiguobacterium*, a chitin-degrading bacterium. The BSFC- diet fed fish had the highest relative abundance of this bacterium, but in digesta of the fish fed the fermented BSF diet (BSFC+P+) this bacterium completely dominated the community, and indicated the presence of dysbiosis, an undesirable state for the gut microbiome. It will be important in future studies to determine, why *Exiguobacterium* has dominated the community for the BSFC+P+ diet, if this bacterium is active and if it provides a digestive function to the host. It will also be very valuable for growing Atlantic salmon aquaculture industry to identify gut bacteria associated with characteristics of optimal production.

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Paper II

Supplementary material

Figure S1. Box plots of alpha diversity measures for the dietary microbial community **A)** The observed richness of ASVs, **B)** Shannon diversity, **C)** Chao1 diversity, **D)** Pielou´s evenness. The P value for each variable is displayed on its respective graph.

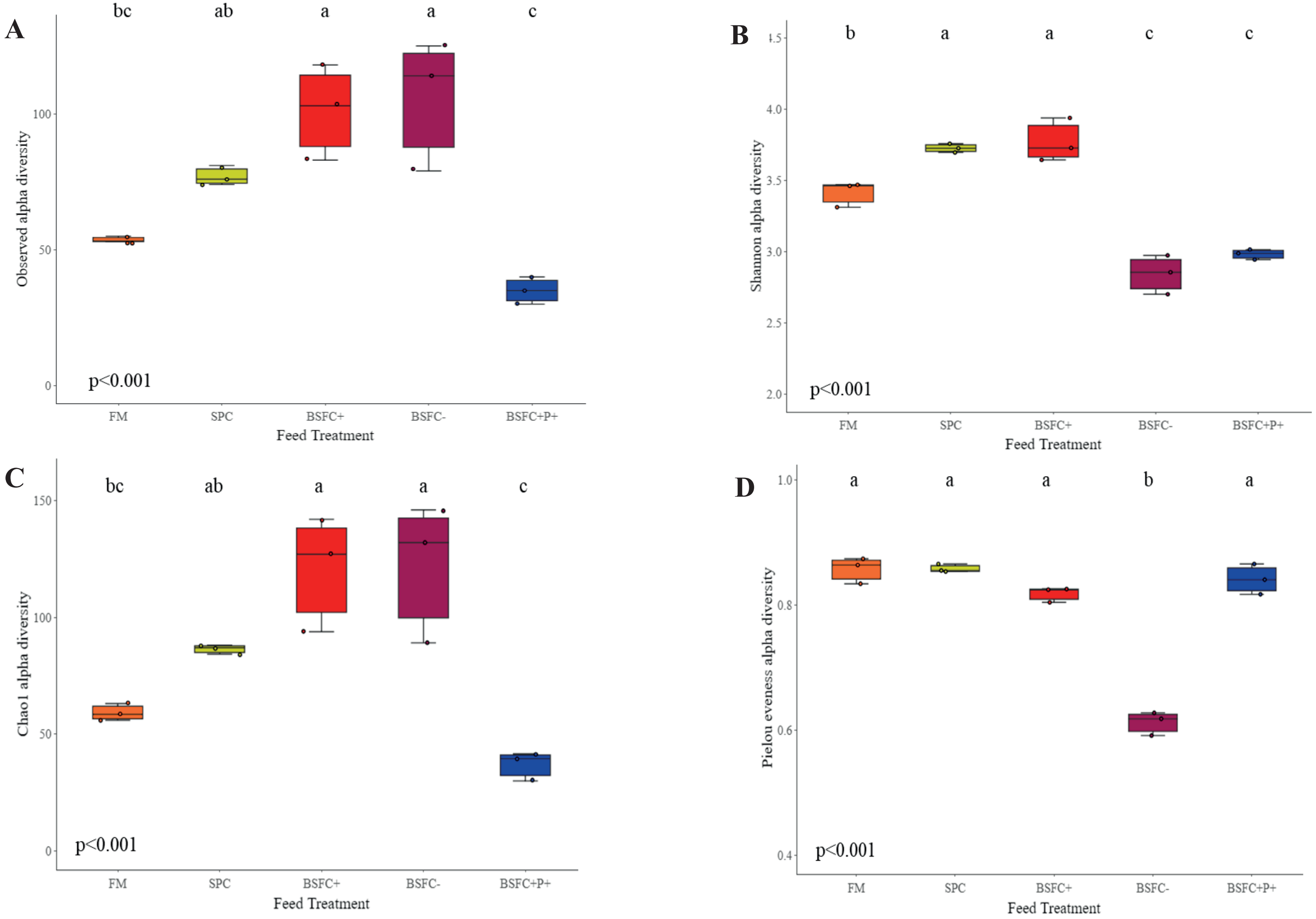


Figure S2. Stacked bar plot of bacterial composition using relative abundance of the most common phyla for the study dietary treatments.

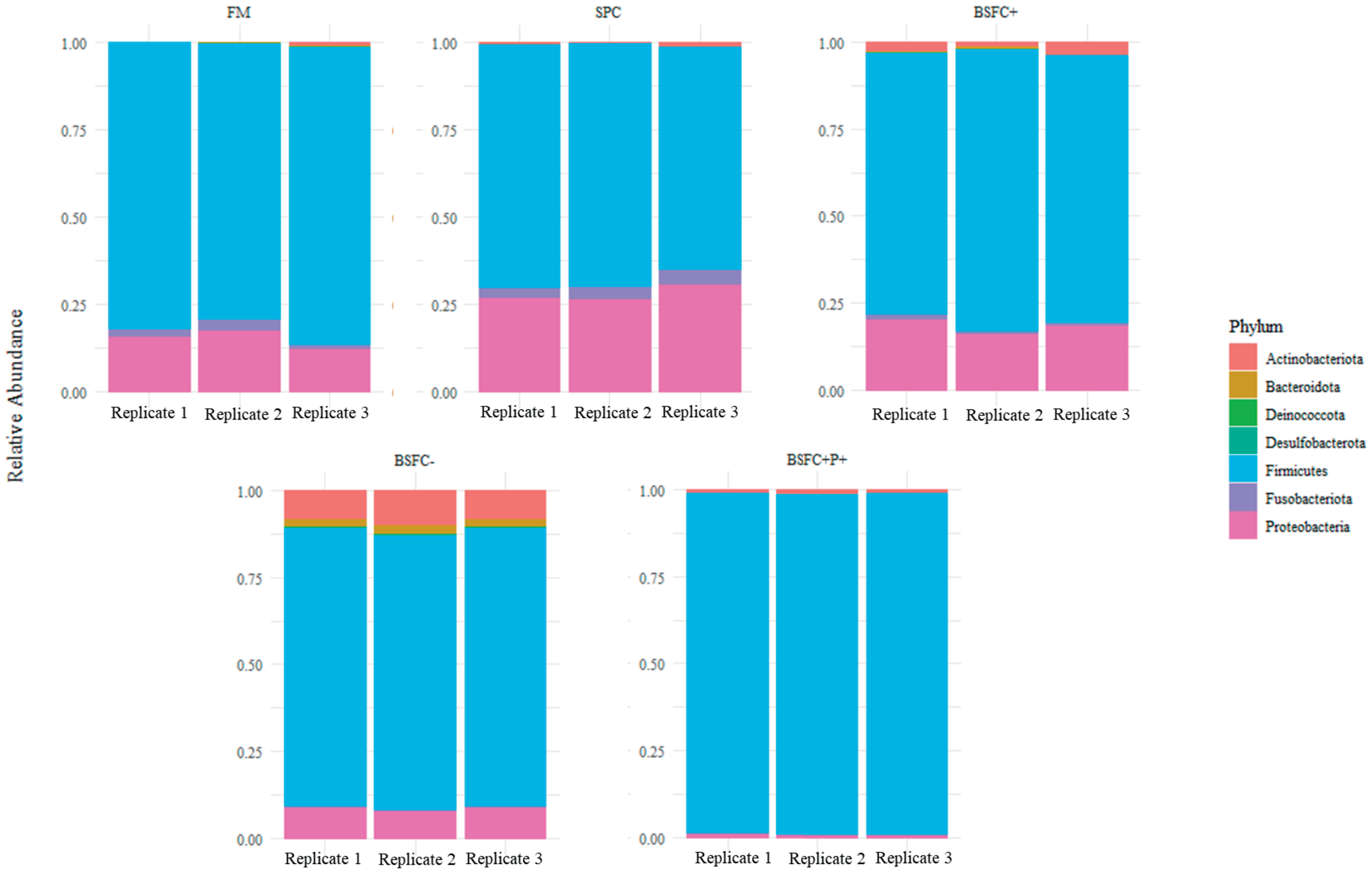
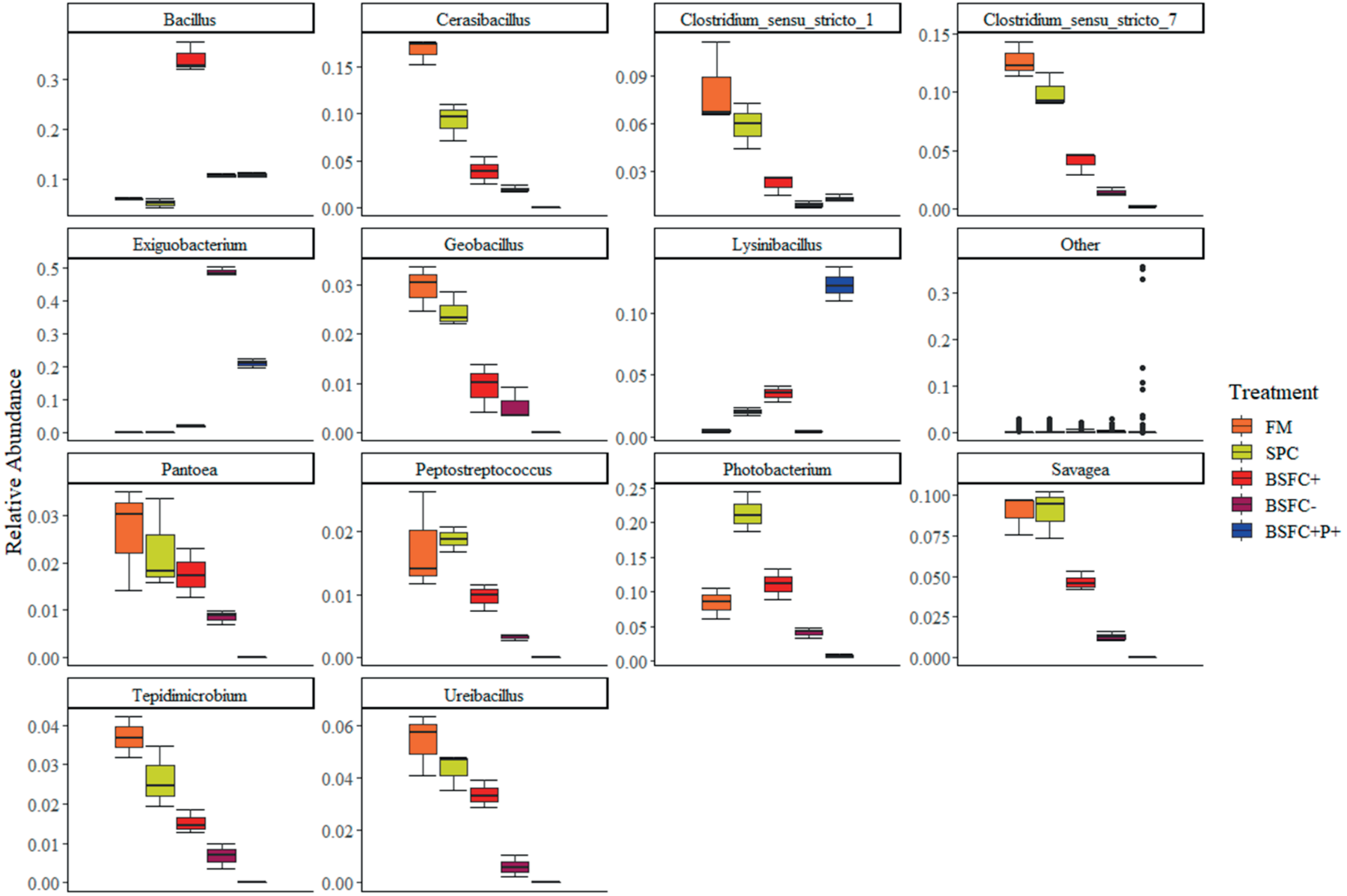


Figure S3. Boxplots of bacterial composition using relative abundance of the most common genera found in each dietary treatment.



Paper III

Torula yeast in the diet of Atlantic salmon (S*almo salar)* and the impact on growth performance and gut microbiome.

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Abstract

Atlantic salmon aquaculture is expanding, and with it, the need to find suitable replacements for conventional protein sources used in formulated feeds. Torula yeast (*Cyberlindnera jadinii*), has been identified as a promising alternative protein for feed and can be sustainably cultivated on lignocellulosic biomasses. The present study investigated the impact of torula yeast on the growth performance and gut microbiome of freshwater Atlantic salmon. A marine protein base diet and a mixed marine and plant protein base diet were tested, where conventional proteins were replaced with increasing inclusion levels of torula yeast, (0%, 10%, 20%). This study demonstrated that 20% torula yeast can replace fish meal without alteration to growth performance while leading to potential benefits for the gut microbiome by increasing the presence of bacteria positively associated with the host. However, when torula yeast replaced plant meal in a mixed protein diet, results suggested that 10% inclusion of yeast produced the best growth performance but at the highest inclusion level, potentially negative changes were observed in the gut microbial community, such as a decrease in lactic acid bacteria. This study supports the continued investigation of torula yeast for Atlantic salmon as a partial replacement for conventional proteins.

Keywords

Lignocellulosic biomasses, *Cyberlindnera jadinii*, torula yeast, Atlantic salmon, aquaculture, gut microbiome.

Background

Aquaculture production is predicted to be increasingly important role in global food security [1]. In Europe, Atlantic salmon (*Salmo salar*) aquaculture continues to grow in both market share and production intensity [2]. Modern formulated feeds have developed from relying heavily on fish meal (FM), primarily sourced from wild capture fisheries, but have evolved to incorporate large quantities of plant proteins sources such as soybean, rapeseed and corn meals in response to economic and environmental pressure[3]. The scale of finfish production now necessitates the incorporation of alternative sources of dietary protein. The next generation of feed ingredients must meet the nutritional requirements of this carnivorous species, while alleviating market competition for terrestrial agriculture products and negating the adverse impact to growth and gut health attributed to anti-nutritional factors (ANFs) present in many plant ingredients [4].

Torula yeast (*Cyberlindnera jadinii,* anamorph name *Candida utilis*) has long been considered for animal feed, historically as a functional feed additive, and more recently as a protein source [5,6]. Existing research has investigated the potential to include torula yeast in diets of poultry [7], pigs [8–10], and a wide range of aquaculture species including, Pacific white shrimp (*Litopenaeus vannamei*) [11], Nile tilapia (*Oreochromis niloticus*) [12] and Atlantic salmon [13,14]. As a protein source, torula yeast has high potential value since it can be cultivated independent of location and climate, and does not add to pressure on existing agricultural systems [5]. It can even be cultivated on side streams such as lignocellulosic biomasses, which are often low-value non-food wastage from other industries like forestry. This, produces ingredients with high protein content and value to aquaculture and could support circular bioeconomy growth [15–17]. Research attention has identified yeast as a promising candidate to replace FM and plant protein sources such as soybean meal (SBM) in feed as demand for alternative protein sources rises [18,19].

The case for inclusion in aquaculture feeds for Atlantic salmon is compelling. Torula yeast has a suitable protein content and amino acid profile for fish feed [5,15,20]. In freshwater (FW) stage Atlantic salmon 28g, torula yeast has successfully replaced up to 40% of the crude protein from FM compared with a control of 58% FM without negative impact on growth performance [18]. Torula yeast has displayed beneficial functional properties such as a reduction in inflammation of the distal intestine during smoltification, a crucial developmental period for Atlantic salmon [14]. Torula yeast has also been associated with immune-modulating benefits which could greatly enhance their value to the aquaculture industry [21]. However, a significant research gap exists in optimising and standardising the dietary inclusion levels which must be addressed to facilitate commercial adoption of this alternative protein [22].

The gut microbiome of cultured fish is understood to play a key role in many aspects of health and welfare, immune development [23] and disease resistance [24], growth [25], digestion and nutrient uptake [26,27]. Multiple factors influence the establishment and final composition of the gut microbiome in farmed Atlantic salmon, one key factor is the diet of the host [25,28,29]. Additional important factors include, the life stage of the fish and the surrounding environment [30,31]. Consequently, it is important to consider changes to gut microbiome composition and their potential consequences when investigating the suitability of new, and alternative feed ingredients for aquaculture [32]. The gut microbiome during early life stages of fish, and the impact of changing diet is of particular interest since it can influence the development of adult gut microbiome and therefore overall development [33,34]. During early life stages, the gut microbiome is highly malleable to dietary influence [35,36]. The presence of certain bacteria have functional immune and development benefits to the fish, for example, lactic acid bacteria (LAB) and *Bacilli* [37], whereas dysbiosis or an unbalanced microbial community is associated with undesirable conditions and consequently, poor health [38,39].

When plant protein replaces or partially replaces marine protein in salmonid diets, the gut microbiome is significantly altered. Pea protein replacing 10% FM increased *Streptococcus*, *Leuconostoc*, *Weissella,* which are LABs [29]*,* and mixed plant meals replacing 97% mixed animal and marine meals increased *Lactocaillales*, *Bacillales* and *Pseudomonadales* [35] in juvenile Rainbow trout (*Oncorhynchus mykiss*). Atlantic salmon post-smolts fed diets containing

30% SBM and soy protein concentrate (SPC) had higher relative abundances of LABs than those fed a FM diet [32]. *T*he yeasts, *Saccharomyces cerevisiae* and *Wickerhamomyces anomalus* replaced 40% and 60% of FM in Rainbow trout (*O. mykiss*) significantly altered gut microbiome, whereas, 20% *S. cerevisae* did not alter the community [40]. In pig diets, torula yeast was associated with selection for *Lactobacillus spp*. in the gut microbiome [41].

There exists a gap in the literature clarifying how torula yeast affects fish gut microbiome despite the growing interest in this protein for dietary inclusion and this is important to characterise the impact of this alternative ingredient for the aquafeed industry [5].

The aim of this study was to evaluate the effect of replacing conventional proteins with increasing inclusion of torula yeast (*C. jadinii*) on both growth performance, and the microbiota present in the gastro-intestinal (GI) tract of juvenile Atlantic salmon. To optimise this characterisation and provide a comprehensive investigation two basal diets were considered. The first, was a marine protein-based diet where fish meal (FM) was replaced with increasing levels of torula yeast to provide a simplified replacement. The second, was a marine protein and plant protein-based combination where a mixture of plant meals (MIX) was replaced with increasing levels of torula yeast to provide a commercial relevant dietary replacement. These two basal diets were formulated separately thus, will be investigated here separately.

2. Methods

2.1. Experimental animals and study design

Atlantic salmon (*S. salar*) hatching on 31 October 2018 were reared at 5.5°C by Stofnfiskur Ltd. (Iceland). Eyed eggs were transferred to Laxar ehf. (Iceland) where they were raised to first feeding using standard commercial techniques and commercial start-feed diet BioMar Inicio-plus (United Kingdom) of 0.5mm pellet size. Fry were transferred to Matís Aquaculture Research Station (MARS) on 23 January 2019, where they were acclimated for one week to the study facilities. All fish within the experiment were individually weighed following a 12-hour fasting period under anaesthetic (2-phenoxyethanol of 300ppm). At the beginning of the feeding trial fish weight was, 1.14 ± 0.1g. Fish were split into eighteen 20L-white circular PVC tanks, in triplicate for each feed treatment. Each tank contained 20 individual fish. Fish were kept in freshwater at 9.5 ± 0.5°C under continuous light (20 ± 4 lux), oxygen levels were maintained above 80% saturation. Fish were fed with the experimental feed treatments for 35 days continuously. The experiment was performed following European and Icelandic guidelines and within the permits and licences of the MARS facility as described in the *Ethical declarations* of this manuscript. The experimental protocols were approved by Matís ohf, Iceland and under the licence FE-1134 (Rekstrarleyfi) from MAST and UST201707 (Starfsleyfi) from the Icelandic Environment Agency. The experimental design also complied with the ARRIVE guidelines.

2.2. Production of torula yeast

Dried, inactive torula yeast (*C. jadinii*) was cultivated by RISE Processum (Borås, Sweden) on wood hydrolysates provided by Arbiom Inc. (Durham, NC, USA). Briefly, wood hydrolysates were generated from hardwood chips (mixed species) locally sourced from Virginia, USA. Subsequently, the hydrolysates were fermented in a 50-L reactor under continuous operation followed by washing, thermal inactivation, and drying to produce the final product.

2.3. Experimental feed treatments and feeding

The two basal diets were formulated for this investigation. In the first basal diet group there were three treatments with a marine protein base, representing a simplified replacement where fish meal (FM) was increasingly replaced with torula yeast with inclusion levels 0% (FM00), 10% (FM10), and 20% (FM20) (Table 1). In the second basal diet group there were three treatments with a marine protein and plant protein combination base, representing a commercially relevant replacement, where a plant protein mixture (MIX) was increasingly replaced with torula yeast with inclusion levels 0% (MIX00), 10% (MIX10), and 20% (MIX20) (Table 2). The chemical composition of all protein sources used in feeds for this experiment from both FM and MIX basal diets are presented (Table 3). The feed treatments were produced by cold pelleting at Matís ohf. (Iceland), with a pasta machine (ADE, Germany). All dry ingredients were milled to bring all materials to equal particle size (IPHARMACHINE, Germany). Dry ingredients were then homogenised in a standard food mixer (KitchenAid, USA) and re-milled to improve the homogeneity of the feed. The dry mix was returned to the food mixer and fish oil was added while simultaneously mixing, a small volume of water was added to produce the ideal consistency for the next stage (200ml). The mix was then processed in the pasta machine to produce 0.5mm pellets. These pellets were dried in a commercial food dryer (Kreuzmayr, Austria) to <10% moisture content. Resulting feed treatments were analysed for chemical composition (Table 1 FM, Table 2 MIX). During the 35-day feeding trial, fish were fed 5 times per day by electric belt-feeder between the hours of 08:00 to 20:00. All tanks were fed identical volumes, with 15% excess based on feed requirements at this developmental stage.

Table 1. Feed formulation and chemical composition for marine protein (FM) feed treatments.

Formulation (g kg^{-1})	***FM0%***	***FM10%***	***FM20%***
Fish Meal [a]	676.8	610.0	542.9
Pre-gelatinised Wheat [b]	209.7	172.9	136.2
Vitamin-Mineral Premix [c]	10.0	10.0	10.0
Fish Oil [a]	103.5	107.1	110.8
Torula yeast (C. jadinii)	0.0	100.0	200.0
Analysed Content (g kg^{-1})			
Dry Matter	935	932	936
Crude Protein	506	502	507
Crude Lipid	162	165	170
Ash	103	100	97
Essential Amino Acids (g kg^{-1})			
Arginine	22.2	27.6	27.9
Histidine	9.3	11.5	11.6
Isoleucine	15.7	19.9	20.7
Leucine	29.3	36.4	37.2
Lysine	29.7	37.4	37.2
Methionine	12.1	12.8	11.3
Phenylalanine	15.5	19.4	19.6
Threonine	17.4	21.4	22.2
Valine	19.5	24.4	25.4
Tryptophan	5.5	5.5	5.2
Non-Essential Amino Acids (g kg^{-1})			
Alanine	23.6	29.2	30.6
Aspartic acid	36.1	43.8	44.9
Glycine	24.9	30.3	30.6

Glutamic acid	56.0	69.8	70.5
Cystein + Cysteine	4.5	5.3	5.2
Tyrosine	12.6	15.7	15.8
Proline	17.0	21.7	19.4
Serine	17.2	21.3	22.4

[a] Laxá hf., Iceland, [b] Emmelev A/S, Denmark, [c] Laxa salmon premix 2006, Trouw Nutrition, Hollands.

Table 2. Feed formulation and chemical composition for combined protein (MIX) feed treatments.

Formulation (g kg^{-1})	***MIX0%***	***MIX10%***	***MIX20%***
Fish Meal [a]	425.0	425.0	425.0
Pre-gelatinised Wheat [b]	191.1	159.4	127.7
Corn Gluten Meal [a]	72.5	53.2	33.9
Vitamin-Mineral Premix [c]	10.0	10.0	10.0
Fish Oil [a]	116.7	116.8	117.0
Torula yeast (C. jadinii)	0.0	100.0	200.0
Lysine-HCl	8.6	6.0	3.4
DL-Methionine	0.4	0.7	1.0
Soy Protein Concentrate [a]	75.7	55.5	35.4
Wheat Gluten Meal [a]	100.0	73.3	46.7
Analysed Content (g kg^{-1})			
Dry Matter	942	918	935
Crude Protein	515	495	506
Crude Lipid	154	162	161
Ash	79	80	86
Essential Amino Acids (g kg^{-1})			
Arginine	25.7	23.5	31.7

Histidine	11.5	10.1	11.6
Isoleucine	20.1	17.6	20.8
Leucine	41.1	35.3	39.0
Lysine	34.4	29.4	32.7
Methionine	11.9	11.1	11.4
Phenylalanine	20.8	18.7	21.4
Threonine	19.6	17.7	20.6
Valine	23.6	21.2	25.3
Tryptophan	5.2	5.2	5.6
Non-Essential Amino Acids (g kg^{-1})			
Alanine	28.0	25.0	29.9
Aspartic acid	40.4	36.0	43.4
Glycine	26.4	24.1	28.2
Glutamic acid	92.9	77.7	81.4
Cystein + Cysteine	6.2	6.1	6.1
Tyrosine	16.6	14.1	17.4
Proline	29.5	24.6	23.6
Serine	22.4	19.8	23.4

[a] Laxá hf., Iceland, [b] Emmelev A/S, Denmark, [c] Laxa salmon premix 2006, Trouw Nutrition, Holland.

Table 3. Chemical composition of the protein used in this study.

	Raw Protein Materials				
Composition (g kg^{-1})	Torula yeast (*C. jadinii*)	Fish meal	Soy protein concentrate	Corn gluten meal	Wheat gluten meal
Dry Matter	938.0	909.0	925.0	910.0	922.0
Crude Protein	514.0	659.0	633.0	582.0	742.0
Crude Lipid	25.1	107.0	2.0	10.0	16.0
Ash	88.4	139.0	90.0	23.0	11.0
Essential Amino Acids (g kg^{-1})					
Arginine	30.8	42.3	42.9	18.6	25.0
Histidine	8.9	18.2	15.5	12.2	14.0
Isoleucine	20.2	30.2	27.5	23.2	24.5
Leucine	32.0	57.1	47.4	96.1	48.3
Lysine	34.2	58.8	37.0	9.1	11.4
Methionine	5.3	19.6	9.0	14.3	11.8
Phenylalanine	18.6	28.9	32.1	37.2	35.7
Threonine	22.9	33.3	25.1	20.3	17.8
Valine	26.0	37.8	28.5	28.0	27.4
Tryptophan	5.8	7.6	8.6	3.1	7.5
Non-Essential Amino Acids (g kg^{-1})					
Alanine	28.4	47.2	26.3	52.7	18.1
Aspartic acid	39.8	69.2	71.3	36.2	21.9
Glycine	21.3	50.3	25.6	17.4	23.3
Glutamic acid	66.1	105.0	114.0	130.0	260.0
Cysteine (+ Cysteine)	3.3	5.8	8.6	11.4	15.5
Tyrosine	16.1	24.6	22.3	30.6	22.9

Proline	15.0	32.1	30.9	55.7	86.9
Serine	21.0	32.6	32.4	33.5	34.7

2.4. Growth Performance

After 35 days of continuous feeding all individual fish from all FM and MIX tanks were weighed (wet weight (g)) and measured (total length (cm) following a 12-hour fast. From this data the Fulton´s Condition Factor (K) and Specific Growth Rate (%) (SGR) over the study period could be calculated: K = (Weight/Total Length^3) × 100 and SGR = ((Ln(Final Weight)-Ln(Initial Weight)) × 100)/t, where t is the number of days over which the trial was run. Mortality was monitored daily throughout the feeding trial.

2.5. Gut Sampling

After the growth performance assessment, all fish were left for one week to recover from handling and fed with the respective FM and MIX feed treatments. At the end of this week fish were fasted for 12hours and then randomly sampled, three (3) fish from each tank, nine (9) fish per feed treatment. Sampled fish were euthanised with a lethal dose of anaesthetic (phenoxyethanol 600ppm) and the fish was rinsed in 90% ethanol followed by sterile distilled water. The GI tract from the top of the mid-gut, just below the pyloric caeca down to the end of the distal gut, was directly removed under sterile conditions, with any content present included. Samples were then stored at -80°C prior to downstream processing.

2.6. DNA extraction, PCR amplification and sequencing

Gut samples were individually homogenised, manually using a sterile petri dish with a sterile scalpel to break up the gut sample. Samples were transferred to a sterile 2ml Eppendorf tube with 300µl of sterile 1mm diameter sterile silica beads (BioSpec Products, United States). 800µl of CD1 solution from the QIAamp PowerFecal Pro DNA kit (QIAGEN, Germany) was added to the Eppendorf tube. Samples were vortexed for 5 seconds and shaken at maximum speed (30Hz) in a laboratory mixer mill (Retsch MM400) for 1 minute. The supernatant (~800µl) was transferred to the PowerBead Pro Tube from the QIAGEN QIAamp PowerFecal Pro DNA kit. The protocol for this DNA extraction kit was then followed and finally DNA was eluted with 80µl of C6 solution. An empty sample with no material was also run to ensure no contamination occurred during the DNA extraction protocol. DNA concentration was measured using 2µl of sample in the Invitrogen Qubit dsDNA BR Assay kit (Invitrogen, Carlsbad, CA, USA), which measured samples with DNA concentration from 2-1000ng. DNA were diluted to 4ng μl^{-1} in a 50µl aliquot. Samples were then subjected to PCR of a region covering V3-V4 regions of the 16S rRNA gene with a universal bacterial primer pair S-D-Bact-0341-b-S-17 (5′−CCTACGGGNGGCWGCAG-3′)/S-D-Bact-0785-a-A-21(5′-GACT- ACHVGGGTATCTAATCC-3′) [42]. The PCR master mix included the diluted DNA, nuclease-free water, Q5 High-Fidelity DNA polymerase (New England Biolabs, Ipswich, USA), Q5 GC Enhancer, 0.5µM of each primer containing Illumina overhang adapters, and 1x Q5 Reaction buffer, 200µM dNTPs (New England Biolabs, Ipswich, USA). Included in the PCR were both positive and negative samples to monitor for successful amplification and absence of contamination of the target region only. The thermocycling protocol had an initial denaturation step (98°C for 30s), then 35 cycles of, denaturation (98°C for 10s), annealing (52°C for 30s), and extension (72°C for 30s), with a final extension (72°C for 2min). Libraries were multiplexed with Nextera XT v2 barcodes (Illumina, USA), normalised using *Sequel-Prep* Normalisation Plates (ThermoFisher Scientific, USA) then sequenced on a MiSeq desktop sequencer (Illumina, USA) using v3 chemistry and 2 × 300 cycles.

2.7. Statistical Methods

2.7.1. Growth performance

Statistical analyses were performed in R version 3.6.1 (2019-07-05). All tests were two-tailed with a significance level set to $\alpha = 0.05$. FM and MIX diets were considered separately since they were formulated independently but identical statistical designs were used.

For growth performance, two dependent variables were statistically assessed, Condition Factor (K) and SGR (%). A Generalised Linear Mixed Model (GLMM) model was assessed first with the package *lme4* [43]. Feed treatment was considered a fixed factor, and tank replicate was considered a random nested factor of feed treatment. For FM and MIX diets the tank replicate did not have a significant effect. Therefore, a Generalised Linear Model (GLM) with the package *nlme* [44] was selected where for FM and MIX diets respectively, feed treatment was a fixed factor and tank effect was not significant. Tukey post-hoc testing was applied to results with significant output.

2.7.2. Gut microbiome

Demultiplexed FASTQ files from Illumina were processed to produce amplicon sequence variants (ASVs) using the *dada2* package version 1.16.0 [45] in Rstudio version 4.0.2 [46]. The function *filterAndTrim* set variables as, *truncLen*=c(280,250), *trimLeft*= 21*, maxN*=0, *maxEE*=c(2,2), *truncQ*=2 and the *learnError* function was performed on a subset of 105888913 reads. The SILVA database version 138 was used to *assignTaxonomy* to the ASVs [47]. The microbial community was analysed using R packages phyloseq [48], microbiome [49] and vegan [50], and visualised with ggplot2 [51]. The number of reads output from the *dada2* pipeline were 15449.44 ± 3575.3 for fish meal-based diets and 13317.48 ± 3341.2 for mix meal-based diets. PCR and DNA negative control samples were included in the sequencing and dada2 pipeline to check for potential contamination of samples, there was no detectable contamination in these control samples. For comparison the read depth was normalised across samples with the function *rarefy_even_depth* to the sample with the lowest read depth. ASVs from the Kingdom *Eukaryota*, Order *Chroloplast* and Family Mitochondria were removed from downstream analysis as they are often remnants from 16S fragments in Eukaryotes and do not belong to the bacteria. Raw 16S rRNA gene amplicon reads are deposited in the Sequence Read Archive (SRA) under BioProject PRJNA732903.

The microbiome community for MIX and FM basal diets were quantitatively analysed using alpha and beta diversity measures. The selected alpha diversity measures were, observed richness of ASVs, Shannon diversity, Chao1 diversity and Pielou´s Evenness. A GLMM was used to assess if there was a significant difference in these alpha diversity measures between the different feed treatments from the FM and MIX diets, respectively. In this model feed treatment was a fixed factor and tank was a nested random factor of feed treatment. The random nested factor of tank was tested by a likelihood ratio test (LRT) [52]. Post-hoc testing was carried out using Tukey test. The microbiome community assemblage for each of the feed treatment types were transformed using a *Bray-Curtis* dissimilarity matrix and non-metric multidimensional scaling was applied. An Analysis of similarity (ANOSIM) test assessd for significant difference between and within fish fed different feed treatments in the FM feeds and the MIX feeds respectively. To further investigate the microbiome community assemblage the relative abundance as a proportion was visualised at the phylum level in stacked bar plots for direct comparison. The genus level was then visualised, with all genera present at less than 1% abundance amalgamated into a category called "Other" and the genera present at greater than 1% abundance were visually displayed using boxplots for each feed treatment in the fish meal-based and mix-meal based treatments respectively.

3. Results

3.1. Growth Performance

For FM diets, fish SGR (%) was not significantly different between the three inclusion levels of torula yeast with very similar average values and standard deviations (FM00:1.09±0.42, FM10: 1.09±0.37, FM20: 1.13±0.39) (Figure 1a). For MIX diets, MIX10 had the highest SGR%. There was a significant difference in fish SGR % between MIX10 and the highest inclusion MIX20. There was no significant difference between the control MIX00 and either MIX10 or MIX20 (MIX00: 1.12±0.39, MIX10: 1.22±0.38, MIX20: 1.03±0.46) (Figure 1b). For FM diets, fish Condition Factor (K) was not significantly different between the three inclusion levels (FM00: 0.85±0.10, FM10: 0.86±0.06, FM20: 0.88±0.09) (Figure 1c). For MIX diets, fish Condition Factor (K) was not significantly different between the three inclusion levels (MIX00: 0.88±0.10, MIX10: 0.87±0.06, MIX20: 0.85±0.06) (Figure 1d).

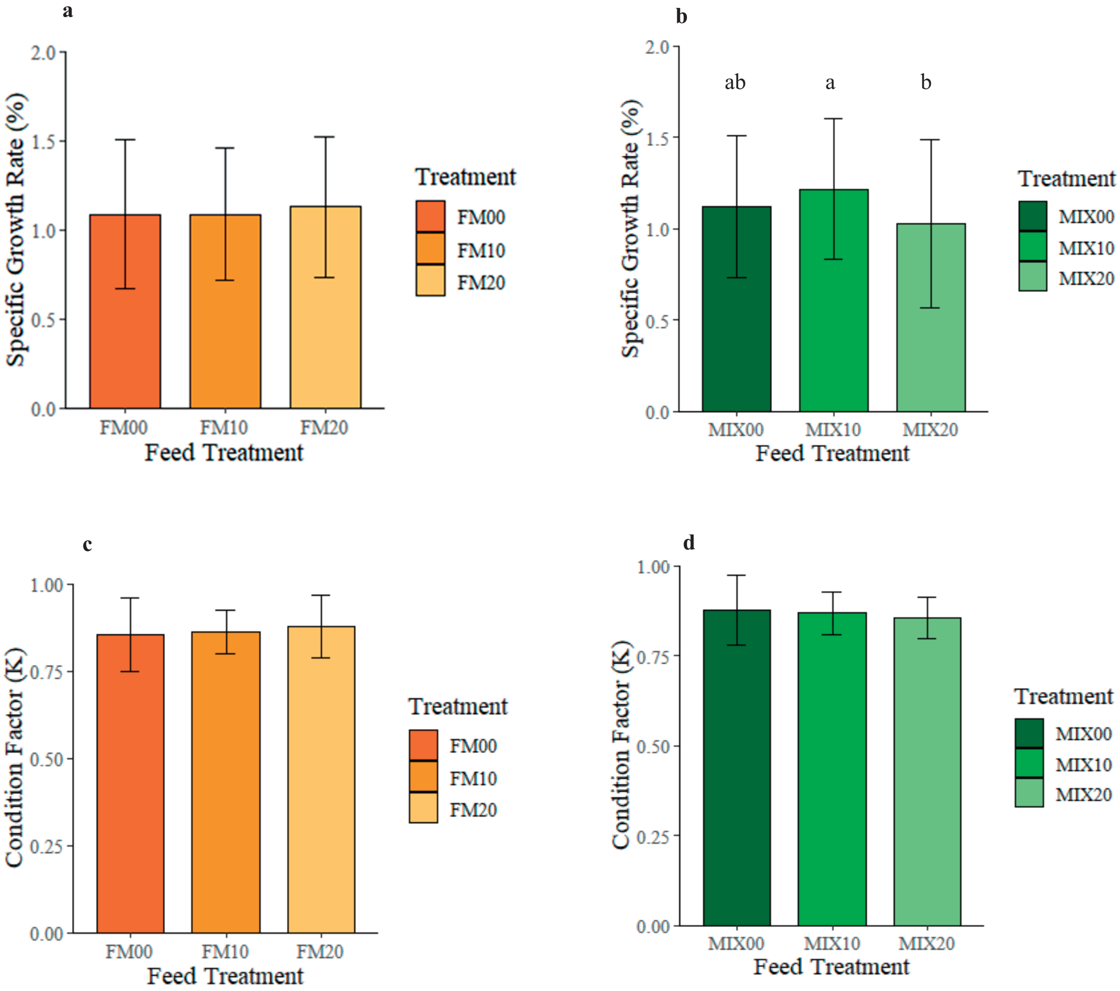

Fig. 1. Bar plots of the SGR (%) for (**a**) FM feed treatments and (**b**) MIX feed treatments, and bar plots of the Condition Factor (K) for (**c**) FM feed treatments and (**d**) MIX feed treatments. Bars represent the standard deviation of the data and different lowercase letters indicate significantly different means ($P < 0.05$).

3.2. Gut Microbiome

For FM diets, no significant difference was detected for any of the alpha diversity measures evaluated across the three inclusion levels of torula yeast. While not significant, FM10 fish did have a higher average value for observed richness (Figure 2a), Shannon diversity (Figure 2b), and Chao1 diversity

(Figure 2c) than other inclusion levels, but FM20 fish had the greatest community evenness (Figure 2d). For MIX basal diets, three of the alpha diversity measures were significantly different. For the observed richness (Figure 3a), Shannon diversity (Figure 3b) and the Chao1 diversity (Figure 3c), there was a significantly lower value for MIX20 than in the control, MIX00. There was no significant difference in the Pielou´s evenness between the three inclusion levels (Figure 3d).

The microbial community composition for fish fed FM diets, were significantly different between all inclusion levels. There was greater difference between inclusion level group than within it, ANOSIM P=0.001 R=0.2234 (Figure 4a). For fish fed MIX diets, the microbiome community composition was significantly different between inclusion levels and there was a great difference between inclusion level than within it, ANOSIM P=0.003 R=0.1097 (Figure 4b). At the phylum taxonomic level for the FM diets, the gut microbiome was dominated by *Firmicutes* for all inclusions of torula yeast (FM00=75.4%±6.3, FM10=85.54%±1.8, FM20=67.43±20.6). The second and third most dominate phyla for all inclusions were *Actinobacteria* (FM00=9.28±6.6, FM10=11.14±2.1, FM20=13.60±5.4) and *Proteobacteria* (FM00=3.07±2.6, FM10=2.44±2.2, FM20=13.11±17.5) respectively. All other phylum occurring were present at 1% or lower relative abundance (Figure 5). At the phylum taxonomic level for the MIX basal diets, the gut microbiome was dominated by *Firmicutes* for all inclusions of torula yeast (MIX00=75.73±13.2, MIX10=77.58±10.2, MIX20=73.78±13.5). The second and third most dominate phyla for all inclusions were *Actinobacteria* (MIX00=10.82±1.9, MIX10=14.08±5.5, MIX20=11.9±5.8) and *Proteobacteria* (MIX00=5.62±2.5, MIX10=4.27± 3.4, MIX20=6.36±9.0) respectively (Figure 5).

At the genus taxonomic level for the FM diets the genus *Staphylococcus* had the highest relative abundance, and abundance was lowest in FM20 compared with FM10 and FM00. For *Clostridium_sensu_stricto1*, *Leuconostoc*, *Preptostreptococcus* and *Paeniclostridium,* there is a pattern of greater average relative abundance in the FM10 inclusion level than both FM00 and FM20. The opposite trend was observed for *Sporanaerobacter*, *Paraclostridium* and *Clostridium_sensu_stricto7*, average relative abundance was slightly lower in FM10 compared with FM00 and FM20. There was decreasing presence of *Tepidmicrobium* with increasing inclusion of torula yeast. There was a similar average relative abundance of *Weisella* for inclusion levels but with a slight increasing trend with increasing torula yeast inclusion (Figure 6). At the genus taxonomic level for the MIX diets the genus *Staphylococcus* had the highest relative abundance, and the average relative abundance increased with increasing inclusion of torula yeast, as did the average abundance of *Weissella*. Conversely, there was a trend of decreasing presence of *Lactobacillus* with increasing inclusion. *Corynebacterium* was highest in MIX10, and *Sporanaerobacter* was lowest in the same inclusion. *Tepidmicrobium* and *Clostridium_sensu_stricto_1* was highest in MIX20. Whereas, for *Peptostreptococcus*, Paraclostridium and *Leuconostoc* they were lowest in MIX20. There were similar levels of *Clostridium_sensu_stricto_7* at all inclusion levels (Figure 7).

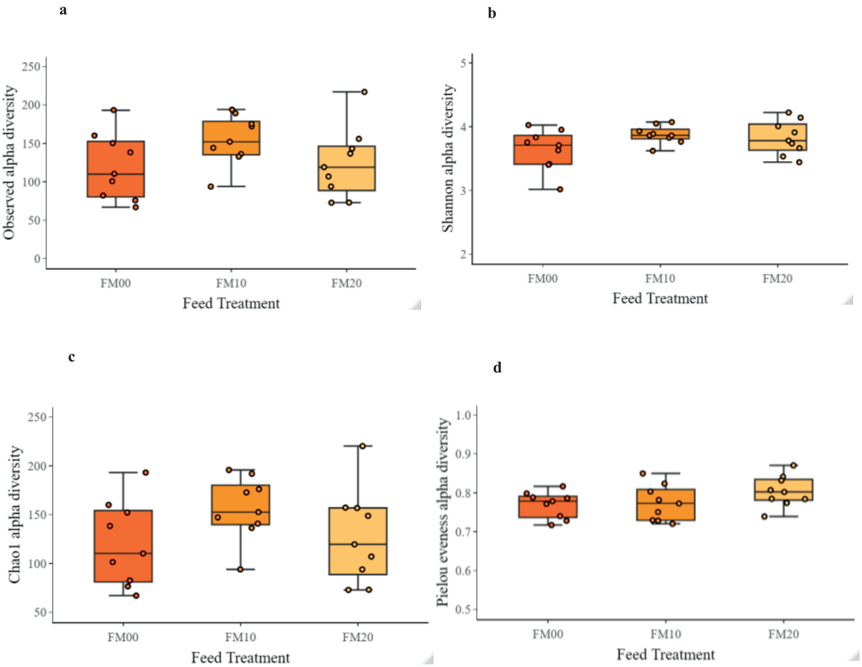

Fig. 2. Box plots of alpha diversity measures for the FM feed treatments **a)** The observed richness, **b)** Shannon diversity, **c)** Chao1 diversity, **d)** Pielou´s evenness.

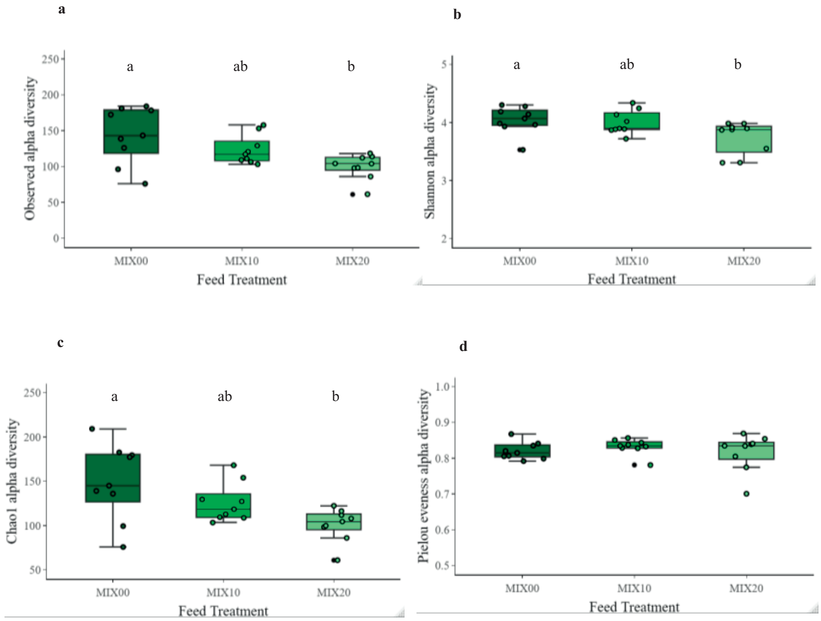

Fig. 3. Box plots of alpha diversity measures for the MIX feed treatments **a)** The observed richness, **b)** Shannon diversity, **c)** Chao1 diversity, **d)** Pielou´s evenness. Different lowercase letters indicate significantly different means ($P < 0.05$).

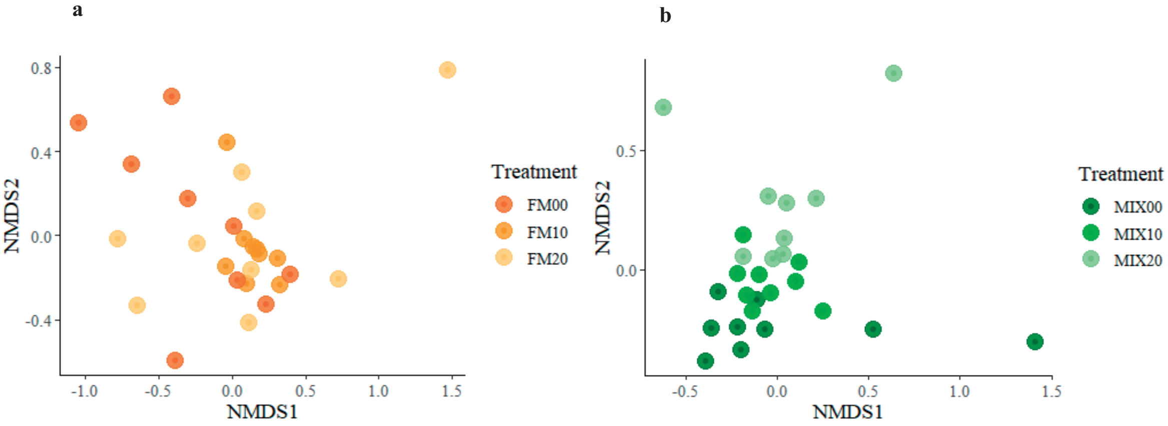

Fig. 4. Non-metric multidimension scaling (NMDS) of fish from **a)** FM feed treatments **b)** MIX feed treatments. Each point represents a single fish and colour indicates the feed treatment inclusion level.

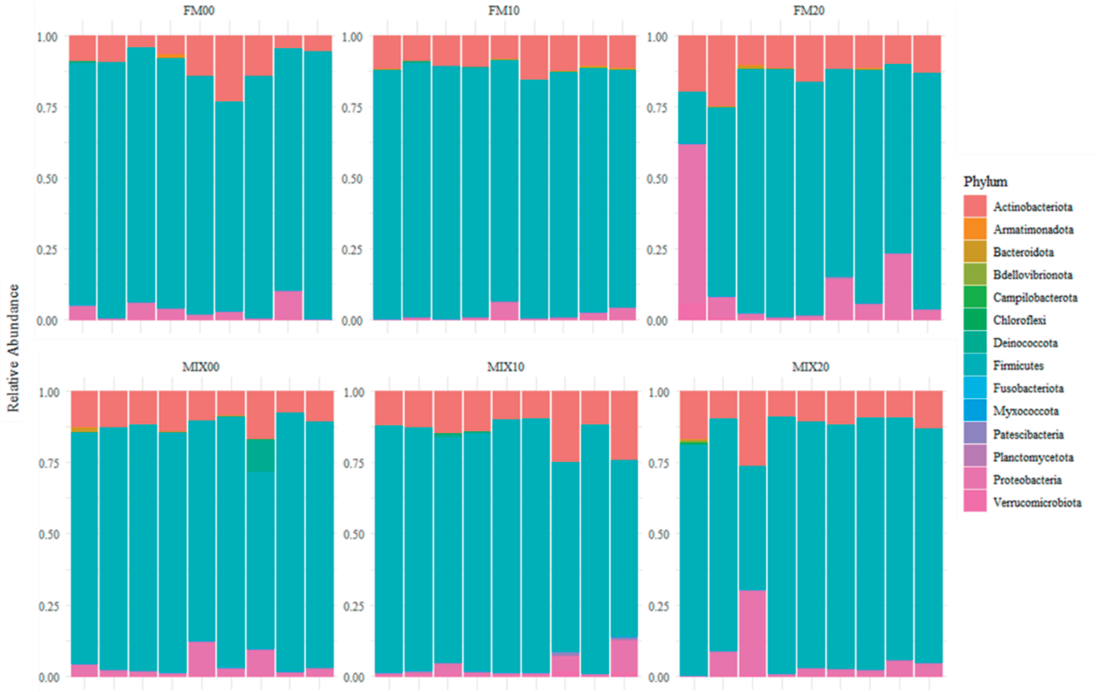

Fig. 5. Stacked bar plot of gut bacterial composition using Relative Abundance of the most common phyla for fish from FM diets and fish from MIX diets.

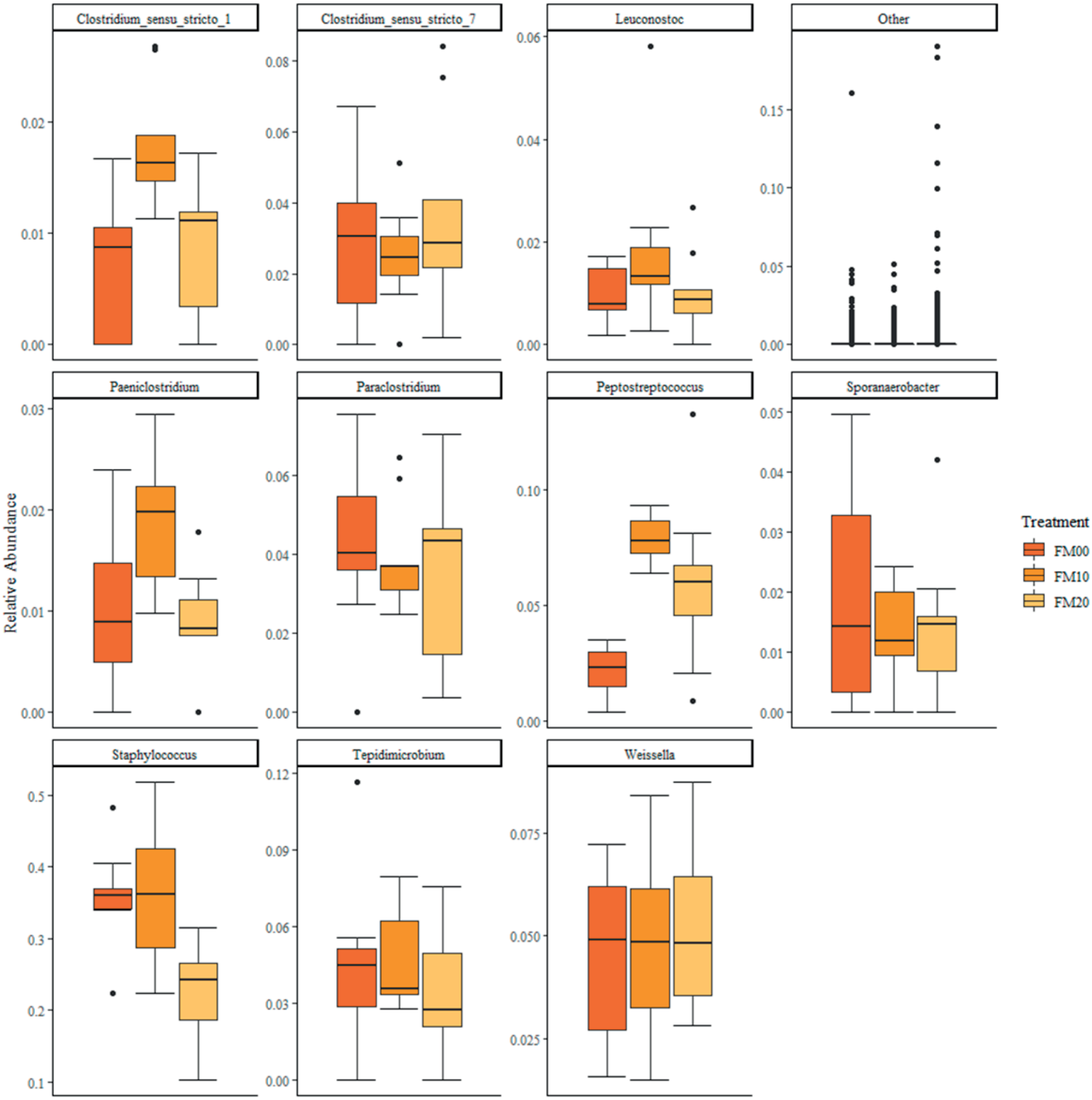

Fig. 6. Boxplots of gut bacterial composition using relative abundance of the most common genera for FM diets.

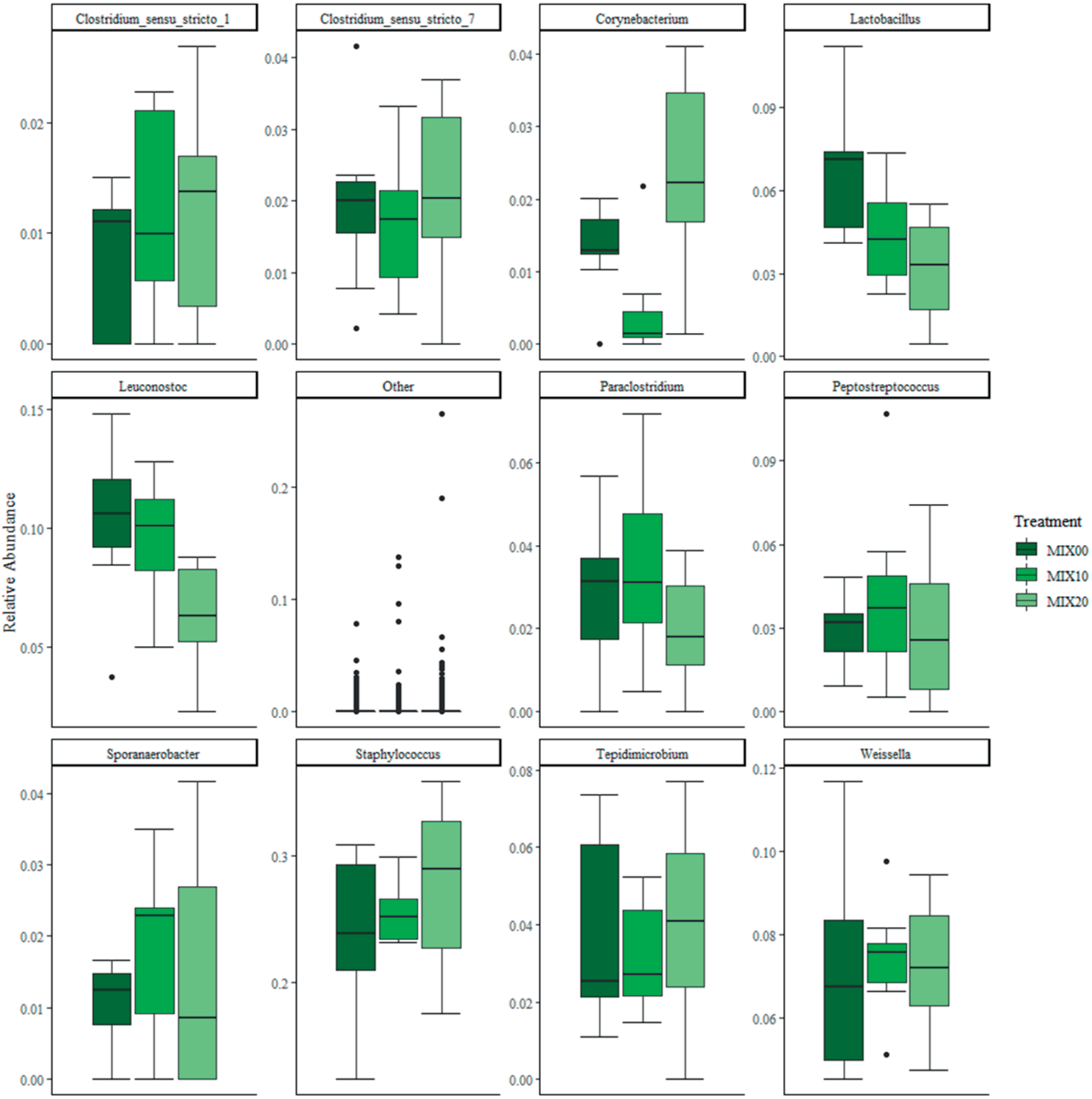

Fig. 7. Boxplots of gut bacterial composition using relative abundance of the most common genera for MIX diets.

4. Discussion

The existing literature has established the potential of torula yeast as a valuable protein source for the aquafeed industry [15,18]. The present study addressed essential knowledge gaps regarding the effect of replacing conventional proteins with increasing inclusions of torula yeast on growth performance and gut microbiome of FW stage Atlantic salmon (*Salmo salar*). Two separate base diets were investigated. A marine protein- based diet where fish meal (FM) was replaced with increasing levels of torula yeast to provide a simplified replacement, and a marine protein and plant protein-based combination where mixed plant meals (MIX) were replaced with increasing levels of torula yeast to provide a commercial relevant dietary replacement.

Growth performance for fish fed the FM diets was comparable between all inclusion levels for both the SGR (%) and Condition Factor (K) up to 20% inclusion. This matches well with existing research where torula yeast has successfully replaced up to 40% of protein from fish meal in the diets of FW Atlantic salmon without adversely impacting growth performance [18]. The addition of torula yeast to a FM diet can even enhance the growth of FW Atlantic salmon[13]. The present study provides further evidence that even in Atlantic salmon less than 2g body weight, torula yeast is a suitable partial replacement for marine protein. The lack of difference in K across the different inclusion levels suggest torula yeast also provides comparable energy levels to marine protein [53]. Growth performance for fish fed MIX diets was best for fish fed the moderate inclusion MIX10, and K was comparable across all diets. Few studies have explored the replacement of plant proteins with torula yeast despite the prevalence of these ingredients in commercial salmonid feed. The reduced growth performance of fish fed the MIX20 compared with MIX10 was also found when torula yeast replaced gluten and starch in a SBM diet at an inclusion of 20% [13], however MIX20 was comparable with the MIX00 control, suggesting MIX10 may be optimal for juvenile salmon. In comparison, when 25% inclusion level of torula yeast replaced a mixture of plant and marine protein sources in Atlantic Salmon through the FW to SW transfer, growth performance and feed intake were improved compared to the control [14]. This difference in results could be due to the strain and growing conditions suggesting this process can be optimised to improve the application for salmonids [18]. In an experiment with Tilapia (*Oreochromis mossambicus*) fed a mixed diet with combined animal, and plant protein, increasing levels of torula yeast showed similar trend to this study, with moderate levels, in this case 30% torula yeast supporting better growth than lower or higher levels [54]. The present study suggests that there is potential for growth benefits with moderate inclusion levels of torula yeast as a replacement for plant protein. The decline in growth performance at higher inclusions levels could be driven by potential over-feeding of yeast presenting a detrimental impact [22], however given that the higher inclusion is comparable with the control, a detrimental impact is unlikely in this study. This is supported by good growth performance of the 20% inclusion for the FM in this study. However, this study along with (Øvrum Hansen) et al., 2019, indicates that higher inclusion levels of torula yeast are more applicable in combination with a marine protein diet than they are with a mixed source protein diet, but that the strain and growth conditions could be improved to optimise utilisation in mixed diets [18].

The gut microbiome of fish fed FM and MIX diets are altered by the replacement of conventional proteins with torula yeast, but the impact trends differ for the different dietary bases. The lack of difference in alpha diversity measures between any of the FM diets suggests that all three diets provide a comparable substrate to support a community with similar defining characteristics. However, the actual community composition established is different, which is shown by the beta diversity, and this is most apparent at the genus level. The MIX diets support gut microbiome communities with different alpha diversity characteristics, especially for the highest inclusion level MIX20, and with different community compositions between the three inclusion levels which are elucidated at the genus level. Compared to the existing literature on the Atlantic salmon gut microbiome during the freshwater stage, the alpha diversity measures for both the FM and MIX fish in this study fall within normal levels for captive individuals [55]. The alpha diversity results of FM diets concur with comparable experiments with other salmonid species. In Rainbow trout (*O. mykiss*) kept in FW, 20% replacement of FM with the yeasts *Saccharomyces cerevisiae* and *Wickerhamomyces anomalus,* did not significantly alter the gut microbial community diversity, but higher replacement levels of 40% and 60% reduced bacterial diversity, and even led to increasing presence of the pathogenic *Candida albicans* at 60% inclusion levels, and reduced the presence of LABs [40]. This suggests that inclusion of greater than 20% of yeasts may be problematic for salmonid health which could in turn affect production even when in combination with marine protein only [22]. To the knowledge of these authors this is the first experiment to assess the gut microbiome of Atlantic salmon when plant proteins in a mixed protein source diet are replaced with a yeast protein source, yet this is a highly relevant concern for commercial salmonid aquaculture. The impact to the alpha diversity is more pronounced than in marine protein diets for observed diversity, Shannon diversity and Chao1 diversity which were all higher in the 0% control and moderate 10% inclusion of torula yeast than in the higher inclusion of 20% in the MIX diets. Higher levels of alpha diversity alone do not necessarily mean a healthier or more resilient community assemblage, and it will be important to confirm resilience through further assessment of health indicators [56]. In early stage Rainbow trout (*O. mykiss),* alpha diversity measures decreased when animal protein was replaced with a mix of plant proteins, at 50% and 97% replacement [35] much higher than in the present study. This indicates changes in diet can be detected in the alpha diversity measures, suggesting that replacing plant proteins with torula yeast in our MIX diets influenced the community, more than replacing FM in a marine protein diet. In another monogastric animal, weaning pigs, a 40% replacement of conventional proteins (a combination of plant and marine proteins) with torula yeast, both the alpha diversity and the beta diversity were significantly altered by the replacement, with lower alpha diversities at 40% replacement [41], suggesting a similar trend as found in the present study for the MIX diets.

Existing research on the gut microbiome of FW Atlantic salmon suggests that even when alpha diversity is not altered, differences may be present in the beta diversity. Chao1 diversity and Shannon diversity and salmon gut microbiome were not altered by acute cold stress or a chronic environmental stress but the beta diversity was altered [57]. In early stage salmonids when the dietary protein composition is altered, existing studies observed a significant shift in the beta diversity and composition of the gut microbiome, in Atlantic salmon [58], in Rainbow trout (*O. mykiss*) [35,59], and in Arctic charr (*Salvelinus alpinus*) [60]. Similarly, in the present study, beta diversity was distinct between each inclusion level of torula yeast irrespective of the dietary base (FM or MIX). Since gut microbiota changes between freshwater and seawater transfer [31], future studies should follow Atlantic salmon fed torula yeast as a dietary protein, from first feeding to harvest to evaluate the impact across different life stages. In other cultured fish species, the presence of yeast species either as a supplement or as a protein replacement for conventional proteins also significantly altered the community compositions, in early-stage zebrafish (*Danio rerio*) [33], in the gilthead sea bream (*Sparus aurata*) [61] and the grass carp (*Ctenopharyngodon idellus*) [62].

The gut microbiome composition of early-stage Atlantic salmon in this study for both FM and MIX diet bases, was similar at the phylum level regardless of the torula yeast inclusion level. Similarly in other studies that have used next generation sequencing in salmonids during the FW growth stages, the dominance of *Firmicutes* followed by *Proteobacteria* have been noted with varying levels of *Actinobacteria* depending on the study [31,40,63,64]. This indicates that a core phyla composition can be expected regardless of diet and influenced by a range of other factors [64]. The drivers of differing community compositions can be seen in this study at the genus level. The dominance of *Firmicutes* at the phyla level for both FM and MIX diets at all inclusion levels is explained by the high relative abundance of *Staphylococcus* at the genus level. This dominance of *Staphyloccocus* species is consistent with other Atlantic salmon and many other fish gut microbiome characterisations [65,66], and it has been associated with nutritional processes in the salmonid, Arctic charr (*Salvelinus alpinus*) [67]. This study revealed different trends for FM and MIX diets, with increasing inclusion of torula yeast further evidence that torula yeast interacts differently with marine protein diets than with mixed source protein diets, this could be to do with potentially differing levels of dietary fibre in FM based diets and the MIX diets in this study. Torula yeast has a high 20% total dietary fibre (TDF), which is higher than FM which is largely devoid of TDF but may see values as high as 5% [68], and may explain why we see a slight trend toward increased diversity indices with increasing torula yeast inclusion, although the effect is minimal. Conversely in the MIX diets, a similarly high TDF from the combined plant meals [68] is being replaced by the torula yeast. This suggests that TDF is not the only driver of the decreased diversity indices seen for the high yeast MIX20 diet. The torula yeast was 12% insoluble fibre and 8% soluble fibre (20% TDF) (R.Ekmay 2021, personal communication 26 May). Future investigations should assess the balance of soluble and insoluble fibre components of formulated feeds with torula yeast and their impact on fish gut microbiota since dietary fibre is known to influence microbiome in other animals [69]. It will be important to elucidate the significance of such differing trends for health and development of cultured salmonids. The LABs from the genus *Weissella* and *Leuconostoc* [37] did not show a strong trend in the FM diets, but slightly increased with increasing inclusion of torula yeast. Conversely, for the MIX diets, the LABs *Lactobacillus*, *Leuconostoc*, and *Weissella* [37] were present, and for the former two they declined with increasing inclusion of torula yeast, whereas the latter increased slightly in the presence of torula yeast. LABs are widely regarded as positive, associated with benefits such as, disease resistance, improved performance, and improved innate immune activities [37,70], however the exact impact is dependent on the species of bacteria and the specific host. In the FM diets of the present study *Clostridium_sensu_stricto_1*, and *Preptostreptococcus* were more abundant in diets containing torula yeast, and highest in the modest 10% inclusion. These genera (*Clostridium* and *Preptostreptococcus*) have been associated with faster growth in Rainbow trout (*O. mykiss*) faecal bacteria samples, although in our study *Clostridium_sensu_stricto_7* did not show a strong trend. However, the genus *Paeniclostridium* was associated with slower growing individuals [71] and this was also highest in FM10 of the fish from this study presenting conflicting findings. Since growth performance was not significantly different in the FM diets of the present study, it might suggest that Atlantic salmon have different bacterial indicators of growth than other salmonids. In the MIX diets the genus *Corynebacterium* was lowest in the moderate 10% inclusion of torula yeast, and highest in the 20% inclusion level, and in Rainbow trout (*O. mykiss*) this genus was associated with slow growing individuals [71] which does correspond with the growth results for the present study of Atlantic salmon. It would be highly valuable for future research to identify bacteria in the digestive tract of Atlantic salmon that are associated with fast and slow growing individuals.

This study indicates that torula yeast can effectively partially replace FM based diet up to an inclusion of 20%, without adverse impact to the gut microbiome community assemblage, and some potential benefits such as, higher abundance of LABs. Conversely, when torula yeast is added to a mix of protein sources it is more effective for growth performance and promoting desirable gut bacteria at moderate levels of 10% inclusion than at the higher inclusion level of 20%. This study sampled the gut digesta and mucosa together, which may have masked further differences in the gut microbiome community composition, since the digesta appears most impacted by diet. Future studies should focus on the digesta where possible [32] although this has proved challenging with very small Atlantic salmon (*Salmo salar*), it would be valuable to investigate the gut microbiome down to the species taxonomic resolution. Additionally this early developmental stage in the salmonid life cycle is a time of a highly dynamic gut microbiome that is malleable and has the potential to change when the dietary input changes and the impact of torula yeast may be different at later developmental stages [30,35].

Conclusions

Torula yeast (*Cyberlindnera jadinii*) has been identified as a promising alternative protein for Atlantic salmon feeds. This study demonstrated that during the freshwater growth stage, torula yeast can partially replace conventional proteins in formulated feeds, but that the optimal level of inclusion may be dependent on the total dietary composition and the types of proteins that are being replaced. In a marine protein diet, this study revealed that 20% torula yeast can be included as a replacement for fish meal without altering growth performance and with possible benefits for gut microbial community such as an increase in some lactic acid bacteria. Comparatively, in a diet that combines marine protein and plant proteins, a 10% inclusion of torula yeast supported better growth performance than conventional proteins. At higher inclusion levels of 20% there were no growth benefits and potentially adverse changes to the gut microbiome, such as a decrease in LABs and increasing levels of bacteria associated with slower growth in other salmonid species. Future research should investigate why torula yeast inclusion produces different results in combination with different dietary proteins, and the potential links between changes in the gut microbiome with growth and resilience in Atlantic salmon.

Ethics declarations and approval for animal experiments

The trial was carried out under the licence FE-1134 (Rekstrarleyfi) from MAST and UST201707 (Starfsleyfi) from the Icelandic Environment Agency. The authors complied with the ARRIVE guidelines.

Consent for publication

Not applicable

Data availability

The datasets generated for gut microbiome during the current study are available in PRJNA732903 repository, http://www.ncbi.nlm.nih.gov/bioproject/732903. Additional datasets used during the current study are available from the corresponding author on reasonable request.

Competing interests

R. Ekmay is employed by Arbiom Inc., a company that develops and markets torula yeast. All other authors declare that they have no competing interests.

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Authors´ contributions

AL conducted the trials, carried out lab work and analysed all data and lead the writing of this manuscript. RE coordinated the production and processing of torula yeast, provide nutritional expertise and contributed to writing and editing of this paper. SK provided expert advice and practical support in gut microbiome sample processing and analysis. SS provided expert advice and practical lab support for gut microbiome sample processing. MV supported animal husbandry and development of gut microbiome protocols. MD supported animal husbandry and development of gut microbiome protocols. BÖS co-ordinated SYLFEED project management and reporting and supported experimental design. JA co-ordinated SYLFEED project management and reporting and supported experimental design and feed formulation, WK provided nutritional expertise and supported experimental design. DB contributed to writing and editing this manuscript and provide experimental, analytical, and academic support. All authors read and approved the final manuscript.

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Paper IV

frontiers

The potential of green and blue protein production systems to support a sustainable, circular bioeconomy in the aquafeed industry

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Abstract

There is a global need to ensure future food security for a growing population. To meet this demand, our food production systems must be resilient, and we must use available resources efficiently and sustainably. Aquaculture is a rapidly expanding food production sector yet producing feed for cultured species represents a significant bottleneck for the industry. Seaweeds, perennial grasses, and perennial legumes are prevalent biomasses in Europe that contribute value through ecosystem services, however they are currently under-utilised. This review maps the ecosystem services provided by these under-utilised production systems and establishes the status of research on these biomasses in circular bioeconomy research and aquafeed research. It then examines the potential to draw these cross-discipline components together and highlights potential for synergy.

Our findings highlight that ecosystem services from seaweed, perennial grass and legume systems are 1) considered highly valuable, 2) that these biomasses have potential value to provide species dependent inputs as proteins or feed additives to the aquaculture industry and 3) that circular bioeconomies are considered a promising mechanism to connect these sectors. A model example is presented of how these under-utilised resources could be sustainably exploited by the aquafeed industry through a circular bioeconomy that supports and boosts valuable ecosystem services. Priorities for future research to implement such a model, research gaps and limitations are discussed.

1. Introduction

World population is rapidly increasing from 7.7 billion in 2019 to an expected 9.7 billion in 2050 (United Nations 2019). This will be paired with a rising food production demand, which will drive an intense competition for natural resources to meet food security needs (Godfray et al., 2010). All this in an era of climate crises (Figueres et al., 2017) characterized by recent new record highs in ocean heat content, sea level rise, and global atmospheric concentrations of greenhouse gases (GHG) (WMO 2020). This creates a need for a more efficient and sustainable use of resources, and dietary transitions to a reduced meat consumption and reliance on animal-derived products (Tilman and Clark 2014; Aleksandrowicz et al., 2016; Poore and Nemecek 2018). The current food supply chain contributes 26% of the total anthropogenic GHG emission (Poore and Nemecek 2018). In Europe, 70% of these emissions come from animal production (European Commission 2020a). Yet 75% of protein ingredients for cultured animal feed are imported, primarily from South America, increasing the impact of the overall supply chain (Westhoek et al., 2011; European Commission 2020b).

Green Protein production systems in the circular economy

Increasing food production is necessary to meet the future demand, yet the potential to expand terrestrial agriculture is limited by a lack of agricultural land. In recent decades, aquaculture has become the fastest growing food production system in the world, a growth trend predicted to continue, providing a promising solution to meet future food security (Godfray et al., 2010). Culturing fish and shellfish is desirable due to high feed conversion ratios, high nutritional content and lower GHG emissions than other food productions systems (Little et al., 2016; Fry et al., 2018). To support continued growth in aquaculture and to improve the sustainability of the industry, it will be necessary to source feed ingredients that minimise environmental impact (Naylor et al., 2009). The aquaculture sector has historically been reliant on wild fish stocks to source fishmeal, particularly for carnivorous species like salmonids (Ytrestøyl et al., 2015; Aas et al., 2019). Environmental as well as economic necessity has led to incorporation of plant-based ingredients to meet the nutritional needs of cultured aquatic species (Gatlin III et al., 2007). Soybean meal has dominated the feed market, but the concurrent intensive crop production puts further pressure on land and water resources, energy usage and competes directly with human and terrestrial livestock. Seaweeds, perennial grasses, and perennial legumes have risen in importance, not only as human food, but also as animal feed and sources of renewable energy (Scordia and Cosentino 2019; van den Burg et al., 2021). As resilient and currently under-utilised biomasses in Europe, recent research efforts are exploring their potential as inputs into circular economic models, such as the use of seaweeds in environmental bioremediation that can later be mined for bioactive components and minerals, or perennial grasses used in bioenergy with residues and waste streams that can be re-valorised for other uses (Lago et al., 2019; Saldarriaga-Hernandez et al., 2020). Recent research has highlighted the potential for these models in the aquafeed industry, particularly as a way of providing critically important sources of high-quality protein (Yarnold et al., 2019).

To successfully exploit proteins derived from seaweed, perennial grass and legume biomasses, upscaling the production and biorefinery processing of the biomass will be important. One possible processing method, which can be applied to both seaweeds, perennial grasses and legumes, is mechanical pressing (Juul et al., 2021; Nielsen 2021), where the fresh biomass is processed using a screw-press, resulting in a fibrous pulp and a juice from which the protein can be precipitated by various methods (Santamaria-Fernandez et al., 2019). This method has been applied for grasses (Corona et al., 2018; Stødkilde et al., 2018; Amer et al., 2020; Nielsen 2021), and seaweed such as *Ulva* spp. (Juul et al., 2021). However, main protein extraction methods investigated for seaweed include alkaline extraction and enzymatic hydrolysis, possibly in a combination (Fleurence et al., 1995; Harnedy and FitzGerald 2013; Abdollahi et al., 2019; Harrysson et al., 2019; Naseri et al., 2020), or removal of other components, such as salt and fiber, thus concentrating the protein in the remaining biomass (Gajaria et al., 2017; Magnusson et al., 2019). Currently, crude protein contents of 50 % in seaweed or grass and legume protein extracts are not unusual (Santamaria-Fernandez et al., 2017; Harrysson et al., 2018; la Cour et al., 2019; Juul et al., In review), the extracts having well-balanced amino acid compositions (Černá 2011; Larsen et al., 2019; Øverland et al., 2019). However, sulphur-containing amino acids are often limiting, e.g. in perennial legumes (Penkov et al., 2003; Apostol et al., 2017; Stødkilde et al., 2018), and *Ulva* spp. (Wong and Cheung 2001; Vieira et al., 2018). The protein extract has higher digestibility compared to the protein in the crude biomass (Bikker et al., 2016; Bleakley and Hayes 2017; Stødkilde et al., 2019), but this might need to be further improved when applied to aquaculture feeds due to a potentially adverse effect from a high amount of residue fibres or phenolic compounds.

The cultivation of seaweeds and perennial land-based species are highly productive systems which contribute to multiple ecosystem services (ES) (Tanneberger and Wichtmann 2011; Joosten 2016; Manevski et al., 2018; Campbell et al., 2020; Jurasinski et al., 2020). ES are increasingly applied as incentives to support nature conservation and as a tool to quantify the tangible values derived from our ecosystems (Vogel et al., 2018). Conventional agriculture on organic soils, can have significant adverse impacts on the soils natural capacity to deliver ES (Joosten et al., 2012), such as carbon dioxide sequestration (Ferre et al., 2019; Günther et al., 2020) and nutrient retention (Joosten 2016; Goriup et al., 2019). Research indicates that biomass production on grasslands and wet organic soils (paludiculture), as well as commercial seaweed cultivation, for protein extraction in biorefineries does not only potentially boost industrial economies, but also nature‘s benefits to society (Wichmann 2017) due to the maintenance of ES delivering ecosystems. Efficient protein extraction from these under-utilised biomasses presents exciting opportunities to both enhance European ES value of these systems and provide novel green and blue proteins to the aquafeed industry (Scarlat et al., 2015).

There is a growing call in aquafeed research to move away from linear supply chains, which are plagued by high levels of waste and contribute to the environmental impact of aquaculture production, towards more innovative, circular economy solutions (Vieira et al., 2020). Recent research efforts have investigated the use of re-valorising forestry side streams, such as lignocellulosic biomasses as a substrate from single cell protein production for aquaculture (Øverland and Skrede 2017; Solberg et al., 2021) highlighting a growing trend for novel aquafeed ingredients from green biomass sources. Circular bioeconomic systems like these are more adaptable to unprecedented change and to develop sustainable practices that can minimise the adverse environmental impacts associated with aquafeed production (Lobell et al., 2008). Circular business models reduce wastage, optimise the use of resources and provide an opportunity to integrate different sectors of industry and society, making them ideal conduits for multi-discipline solutions to global problems such as climate change, food security and food waste management (Mak et al., 2020; Vieira et al., 2020). Development of innovative non-linear economic models making novel proteins from underutilised biomasses accessible to the European aquaculture industry both support market innovation (Hasselström et al., 2020) and are compatible with existing policy drivers such as European directives (e.g., 2008/56/EC European Marine Strategy Framework Directive (European Commission 2008), Blue Growth Strategy (European Commission 2017a)). It will be vital to collate existing knowledge across research fields of aquafeed development, green-blue biomass production, ecosystem services provided by these systems, and circular economies to efficiently solve multiple issues and to improve the sustainability of our highly pressurized food production systems.

This review has four key objectives: 1) to map ecosystem services provided by under-utilised green and blue production systems, 2) to assess how these biomasses and proteins derived from these systems are currently incorporated into the circular bioeconomy research, 3) how they are currently incorporated into the aquafeed industry, and lastly 4) to highlight the potential for cross-disciplinary synergy and future research needs.

2. Methodology

For this review, a meta-synthesis approach was applied, integrating results from a wide range of studies across different research disciplines. The existing research literature was reviewed in the three key categories according to the objectives of this review to best understand the existing connections in the topics of interest: 1) underutilised green and blue biomasses and ecosystem services (ES), 2) novel proteins from underutilised green and blue biomasses and the circular bioeconomy, 3) novel proteins from underutilised green and blue biomasses and aquafeeds. This review considers blue and green proteins to be those proteins derived from seaweeds, perennials grasses and perennial legumes. Selected keywords (Section 1 of Supplementary Material) were used in two search engines, Scopus and Web of Science, with no publication date limit and with a pre-selection of excluded words, yielding non-relevant results (Table 1). The ES searched for in category (1) of this review were selected based on extensive knowledge of those ES associated with blue and green production systems and are listed in Table 2. The keyword search yielded 853 and 830 articles in Scopus and Web of Science (Figure 1), respectively on 15.09.2020. The articles found in both search engines were entered into a bibliographic software program (EndNote©) for removal of duplicates. Further, titles and abstracts were screened, and non-relevant articles, and articles that only referred to keywords less than twice without related content were discarded (Figure 1). Subsequently, full texts of eligible articles were examined, and a second selection performed. This resulted in a total of 180 eligible articles reviewed (Section 2 of the Supplementary Material), representing 11 % of the initially obtained results. To visualise the prevalence of keywords retrieved by the search engines, a word-cloud was generated (http://www.wordart.com) where the more prevalent a word is, the larger the word will appear (Figure 2).

The eligible articles (Section 2 of the Supplementary Material) were information-mined to address the research objectives of this review. For category (1), quantitative information was collected on the ES attributed to three specific biomass production systems: seaweed cultivation, permanent grassland and agriculturally used peatlands. For category (2), information was collected on the presence of these different production systems and the proteins derived from them in circular bioeconomy research, including an examination of potential challenges regarding the integration of these. For category (3), information was collected on the application of the different underutilised biomasses assessed in this meta-synthesis, and the blue and green proteins derived from them, exploring how they are used in aquafeed.

Table 1. Excluded terms in the search-string, and exclusion criteria for processes of screening and eligibility assessment applied in each of the meta-synthesis categories.

Excluded Terms	Exclusion Criteria		
	Ecosystem Services	*Economy*	*Aquafeed*
Human*	Cyanobacteria	Annual plants	Insects
Insect*	Tropics	Insects	IMTA
Genetic*	Sphagnum	Wastewater	Seaweed farming
Legume*	Wastewater	Herbicides	Non-aquatic anima
Soy*	Insects	Silviculture	Not in English
Supplement*	Lidar	Beans	Beans
Poultry*	Remote sensing	Not in English, Danish, German	Fish farm waste
Micro*	Malaysia	Conference papers	Annual plants
	Indonesia	Bioenergy products	Yeast
	Silviculture	Mineral biofortification	Conference papers
	Constructed wetlands	Lactic-acid bacteria	
	Catch crops	Animal husbandry	
	Chinese articles	Soil fertility	
	Conference papers	"Economy" as buzz word	

Table 2. Types of ecosystem services, according to the Millennium Ecosystem Assessment (2005), assessed in the meta-synthesis.

Indirect benefits to humans		Direct benefits to humans	
Supporting	*Regulating*	*Provisioning*	*Cultural*
Nutrient Cycling	Bioremediation	Biomass (Food, Energy, Raw Materials)	Educational and Scientific
Soil Formation	Water Purification	Water	Recreational
Habitat Provision	Pollination	Medicine	Spiritual
Biodiversity	Pest control		Aesthetics
	Flood Control		
	Erosion mitigation		
	Nutrient Retention		
	Carbon & Climate		

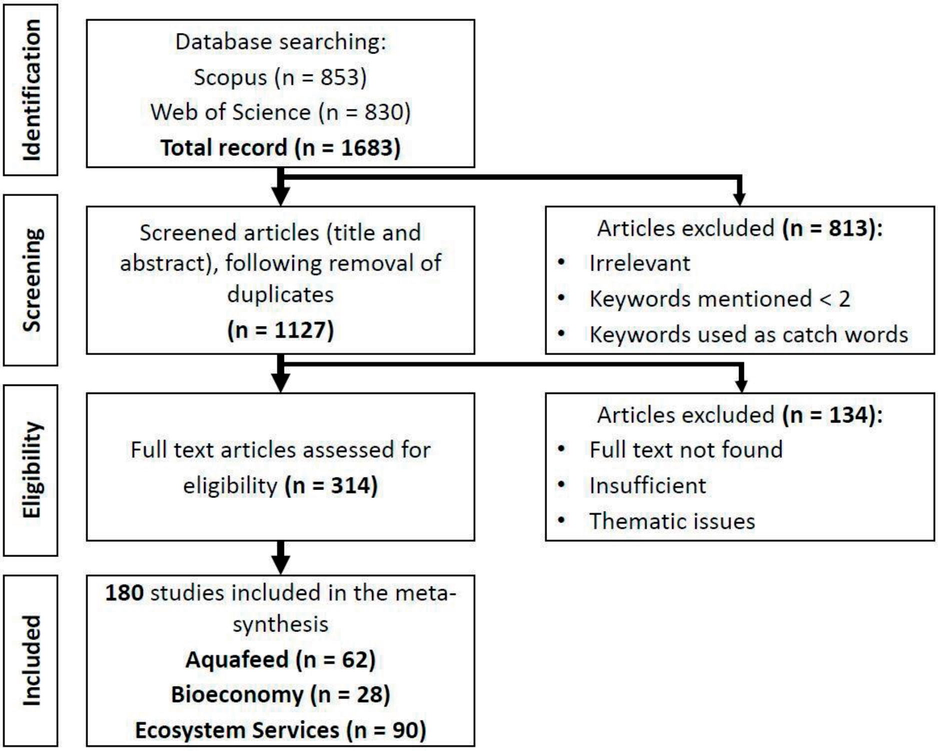

Figure 1. Protocol followed to retrieve material for this review. This process resulted in 180 studies included for analysis.

3. Results

3.1. Keyword word cloud

The word-cloud shows the most prevalent words retrieved in the search of the literature were ecosystem services, growth, fish, management, carbon, meal, peatland, feed, and water (Figure 2).

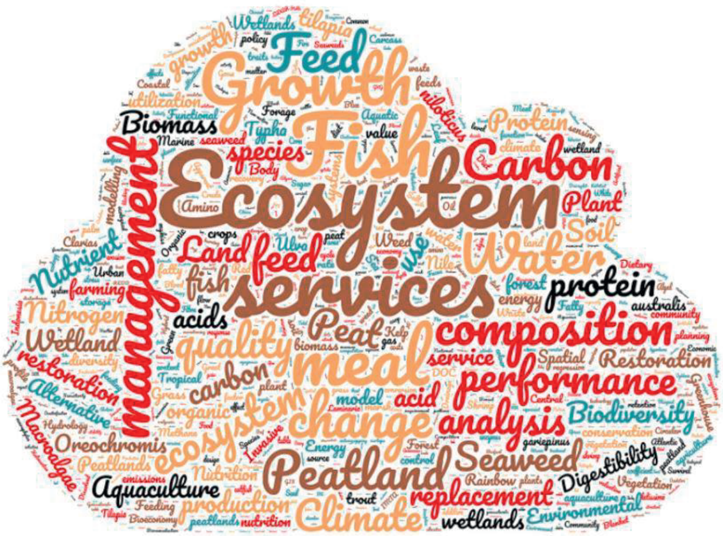

Figure 2. Word cloud visualising the prevalence of keyword retrieved by search engines.

3.2. Mapping Ecosystem Services

A total of 90 articles regarding ES on green and blue protein production systems met the review criteria. 89 % of screened articles discussed multiple ES. Articles on peatland ES were most frequent and spanning the longest timeframe (2005 – 2020), whereas ES provided by seaweed cultivation are, with the earliest reviewed publication from 2013, a relatively recent notion (Figure 3).

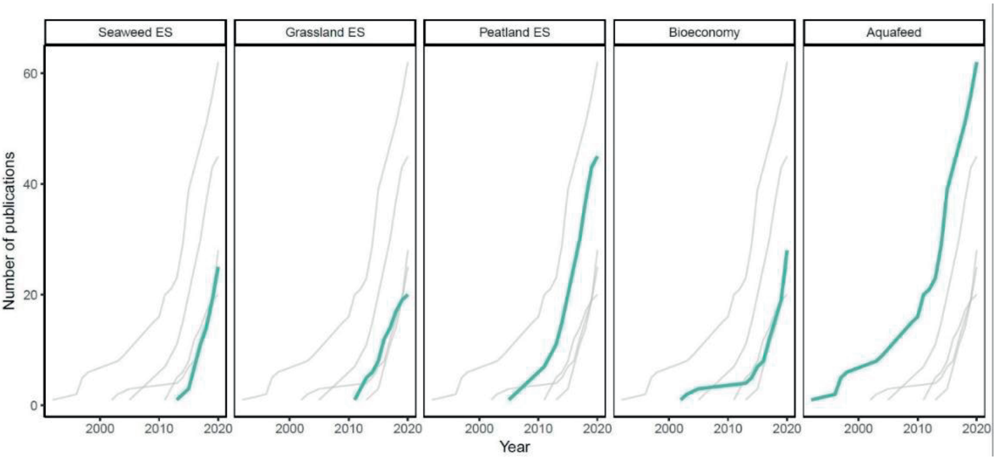

Figure 3. Number of publications over time for each of the review fields. Ecosystem services (ES) are separated into each protein-producing ecosystem to reflect individual changes in research and application development.

3.2.1) Seaweed production systems

ES related to seaweed cultivation were mentioned in twenty-five (n = 25) articles. Regulating services made up, with 41 statements, the majority of reported ES (Figure 4A). Most notably, carbon and climate regulating, as well as nutrient retention (n = 15, and n = 17, respectively) were highlighted as crucial ES provided by seaweed cultivation. Provision of biomass as a provisioning service of seaweed cultivation for the combined categories of food, energy feedstock, and raw materials were the only example of provisioning ES mentioned (n = 17). Habitat provision (n = 10) and recreational aspects (n= 6) within the ES categories of supporting and cultural services were frequently mentioned in the reviewed literature. Seaweed, and particularly kelp species (e.g., in Graiff et al., (2015)), were associated with high rates of inorganic carbon accumulation, containing approximately 30% C kg^{-1} DM (Vondolia et al., 2020). Carbon fixation rates by cultivated kelp have been reported with 1.2 – 1.8 t ha^{-1} yr^{-1} (Smale et al., 2013; Kim et al., 2015; Augyte et al., 2017; Duan et al., 2019), accumulating atmospheric carbon (760,000 t yr^{-1}), and mitigating 2.8 million t yr^{-1} of carbon dioxide emissions from benthic sediments (Kim et al., 2017). Further, natural macroalgae beds (Layton et al., 2020a) may contribute with an unknown share in deep water carbon sequestration, referred to as *blue carbon* (e.g. (Hasselstrom et al., 2018; Layton et al., 2020b)), due to their massive biomass repository (van Son et al., 2020). In addition, the ability of several macroalgae species to use HCO_3^- as a source of carbon, and hence mitigate ocean acidification, was highlighted (Duan et al., 2019; Schoenrock et al., 2020). Nutrient retention on the examples of nitrogen (N), phosphorus (P), as well examples of heavy metal bioremediation (Layton et al., 2020) were mentioned as significantly mitigating eutrophication (e.g. Zhang and Thomsen (2019)) and playing a key role in offshore nutrient cycling (Lind and Konar 2017). Average reported N concentrations in tissue of the most common cultivated seaweeds ranged between 1.7 % N (*Eukema* spp.) and 6.7 % N (*Saccharina* spp.) (Bruhn et al., 2016; Kim et al., 2017). For seaweed biomass, harvestable amounts of nutrients ranged between 88.7–274 kg N ha^{-1} yr^{-1} (Kim et al., 2015; Augyte et al., 2017) and 12–14.5 kg P ha^{-1} yr^{-1} (Hasselström et al., 2018). The highest values were reported under high environmental nutrient availability in aquatic systems (Boderskov et al., 2016; Bruhn et al., 2016). The results imply a global retention potential of about 1.2 million t N yr^{-1}, and 0.07 million t P yr^{-1} when considering the production rate of 30.1 million t of seaweed in 2016 (de Queiroz Andrade et al., 2020). Further, positive implications of nutrient retention by seaweed farming in proximity to fish aquaculture were highlighted in the reviewed literature (e.g. Bruhn et al., (2016) and Vondolia et al., (2020)).

3.2.2) Grassland production systems

ES within grass production systems on mineral soils were mentioned in twenty (n = 20) articles. Overall, regulating services were, with thirty-nine (n = 39) statements, most associated with grassland production systems, followed by provisioning, supporting and cultural ES (Figure 4 B). Most notably, carbon and climate regulation, as well as erosion mitigation (n = 13 and n = 8, respectively) were highlighted as crucial ES provided by grass cultivation. Biomass use as the fundamental driver for grassland cultivation was mentioned thirteen times (n = 13) in the category of provisioning services. Further, regulating services and supporting services of nutrient retention (n = 5) and nutrient cycling (n = 4) were highlighted. Water, biodiversity and habitat, as well as cultural aspects of ES on grasslands were considered secondarily to other more prioritised ES. The majority of reviewed articles stated biomass provision and carbon and climate regulation as the key ES of grasslands due to the functionality of roots, their depth, and abundance for the build-up of soil organic matter (SOM) (e.g. Barkaoui et al., (2016)), which is correlated with increasing biomass yields (Meyer et al., 2015). Carbon sequestration, at a rate between 0.30.5 t C ha^{-1} yr^{-1} in soils with low initial soil organic carbon (SOC) contents (Meyer et al., 2015), was reported to be higher in deep rooted plant systems (e.g., *Festuca* spp. (Cougnon et al., 2017) as compared to perennial legumes and lignin-poorer grasses (e.g. *Lolium perenne* (Reheul et al., 2013). Further, increased soil carbon sequestration on permanent grasslands was highlighted to reduce emissions of CO_2 (Griffith et al., 2011; Lewandowski 2013; Everson and Everson 2016; Castelli et al., 2017; Kayser et al., 2018; Qi et al., 2018; Bengtsson et al., 2019) as well as N_2O (Abalos et al., 2014), mitigating adverse impacts on the climate. In addition, harvest of grass biomass has the potential to remove N loads in soil by 4 kg N ha^{-1} yr^{-1} (Wüstemann et al., 2014) to 77kg N ha^{-1} yr^{-1} (Meyer et al., 2015), depending on species.

3.2.3) Agriculturally used wet peatlands

The literature search on ES provided by wet peatlands under agricultural management, known as paludiculture, resulted in 45 (n = 45) articles. Regulating services were stated most (Figure 4 C) in the reviewed literature, with (n= 135) mentions, followed by provisioning and supporting services (n = 56 each) as well as cultural services (n =26). Most notably, carbon and climate regulation (n = 39), biomass provision (n = 36), nutrient retention (n =26) and combined water related services, independent of ES category (n = 66), were highlighted as crucial ES provided by cultivated wet peat soils. Further, cultural services and, in particular educational and scientific services (n = 9), were frequently mentioned. Agriculturally used wet peatlands store about 30 % of the global C pool (Alderson et al., 2019), due to root exudates (Bacon et al., 2017) and the accumulation of plant litter under anoxic and waterlogged conditions (Bonn et al., 2016). Further, the mitigation of GHG emissions, mainly CO_2, CH_4, and N_2O, were reported with 10 (Wüstemann et al., 2014) to 60 t CO_2-eq ha^{-1} yr^{-1} (Geurts et al., 2019), while at the same time significantly contributing to soil formation (e.g. Rebhann et al., (2016) and Ferré et al., (2019)). Peatland-derived grass biomass, also named paludicrops, and in particular, *Phalaris* spp. (Tasset et al., 2019) and *Typha* spp. (Mitsch et al., 2013), were associated with high rates of carbon fixation by 116-209 g C m^2 yr^{-1} (Billett et al., 2010; Kareksela et al., 2015). Nutrient retention on the examples of N, P, and heavy metal bioremediation, were frequently mentioned as a critical regulating service by agriculturally used peatlands. Wüstemann et al. (2014) highlighted highest de-nitrification benefits per hectare, while e.g. Steffenhagen et al., (2010) and

Lennartz and Liu (2019) mentioned wet cultivated peatlands as being the most efficient ecosystem regarding storage of nutrients within plant tissue. *Phalaris* spp. outperformed *Typha* spp. and *Phragmites* spp. efficiencies regarding soil nutrient removal by biomass harvest (Martina et al., 2014), with removal rates between 50–200% of excess N and P applied by fertiliser (Jakubowski et al., 2010; Vroom et al., 2018), or 9.6 g N m^2 yr^{-1} and 30-60 g P ha^{-1} yr^{-1} (Bansal et al., 2019). Similarly high removal rates were reported for water purification by paludicrops (Bonn et al., 2014; Kotowski et al., 2016; Kløve et al., 2017; Finlayson and Milton 2018), highlighted by up to 20 900 (N) and eight times (P) higher contents within plant tissue than in bodies of open water for long-duration biomass stands (Zerbe et al., 2013). High energy values by combustion for biomass, equivalent to 0.4 t of fossil oil per t of biomass input were reported in the reviewed literature (Carson et al., 2018).

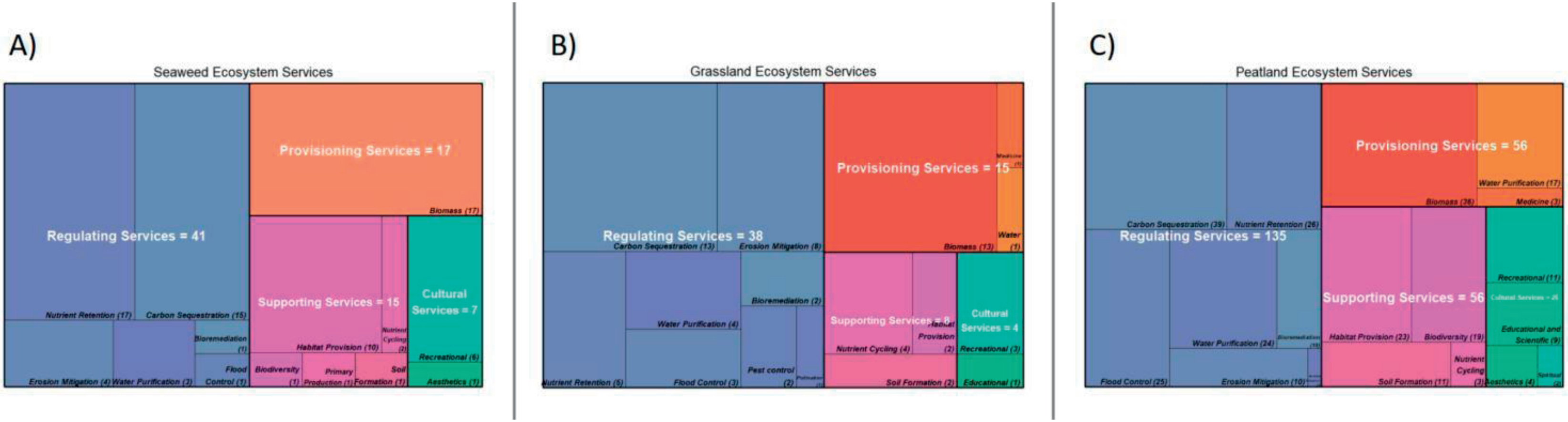

Figure 4. The Ecosystem Services detected in the literature included in this review for, **(A)** seaweed production systems, **(B)** grassland production systems, **(C)** agriculturally used wet peatlands.

3.3. Application in circular bioeconomy

For seaweed, perennial grasses, and legumes, “circular economy” and “bioeconomy” or like terms are frequently mentioned. However, few of these papers investigate actual circular bioeconomic modelling of the biomasses. Twenty-eight (n = 28, Figure 3) papers were found relevant during the screening process. Of the 28 papers, 16 (n = 16) had bioeconomy as the main subject (Figure 5), either making a bioeconomic model, an economic analysis or an analysis of the potential of the biomass to be incorporated in a circular bioeconomic model. The remaining papers were primarily on either biomass cultivation or biorefinery but discussing the perspectives for bioeconomy of the respective topics. Half (n = 8) of the articles having bioeconomy as the main subject, had a secondary focus on biomass cultivation. Seventeen (n= 17) out of 28 articles were on seaweed. None of the 28 articles were published before this century, 21 (n = 21) of the articles being published within the last five years (2016-2020). Nineteen (n = 19) of the articles had authors based in Europe at the time of publication. Ten (n = 10) articles touched upon protein as a biomass constituent or as part of biomass biorefinery. The literature, however, is very limited regarding the protein production and extraction in a circular and/or bioeconomic context. When protein is mentioned, it is merely as a part of a biorefinery cascade, a potential product from the biomasses. One (n = 1) paper explicitly takes a closer look into the economy of protein production from seaweed and calculates the price of protein extracted from *Kappaphycus* sp. to 5000 USD t^{-1}, estimating a price range of 1000-15,000 USD t^{-1} (Golberg et al., 2019).

The main characteristics of opportunities mentioned for the biomasses in a circular bioeconomic context in the reviewed articles couples to ecosystem services. Seventeen (seaweed: n = 8, perennials: n = 9) of the articles mention either directly or indirectly ecosystem services provided by the cultivation of biomass, especially supporting, and regulating services, such as nutrient cycling, increased biodiversity, bioremediation, and carbon sequestration. Moreover, the seaweeds and perennials are stated to be under-utilised, having high productivity and high nutrient efficiency (seaweed: n = 4, perennials: n = 3), implicitly stating the high potential of the biomasses in a bioeconomic model. For seaweed, the benefit of independence of fresh water and arable land is moreover mentioned in four (n = 4) articles. Ten articles (seaweed: n = 6, perennials: n = 4) mention either the biomass in a circular economy context or the possibility to use the biomass waste. The potentials for circular bioeconomy are especially connected to nutrient cycling (supporting ecosystem services) (seaweed: n = 3, perennials: n = 1), and a zero-waste strategy (seaweed: n = 3, perennials: n = 2), using residual biomass as e.g., energy feedstock or bio-sorbent.

Regarding challenges, nine articles (seaweeds: n = 5, perennials: n = 4) directly mention either a lack of political drivers, governmental support, and/or mismatches between policy narratives and political frameworks, development processes and potentials. Moreover, an often-mentioned challenge (seaweed: n = 4, perennials: n = 3) is the need for efficient biorefinery technologies, upscaling laboratory processing methods as well as harvesting technologies. For seaweed another challenge is the varying chemical composition and yield of the biomass (n = 3), depending on e.g. species, season and geographical location.

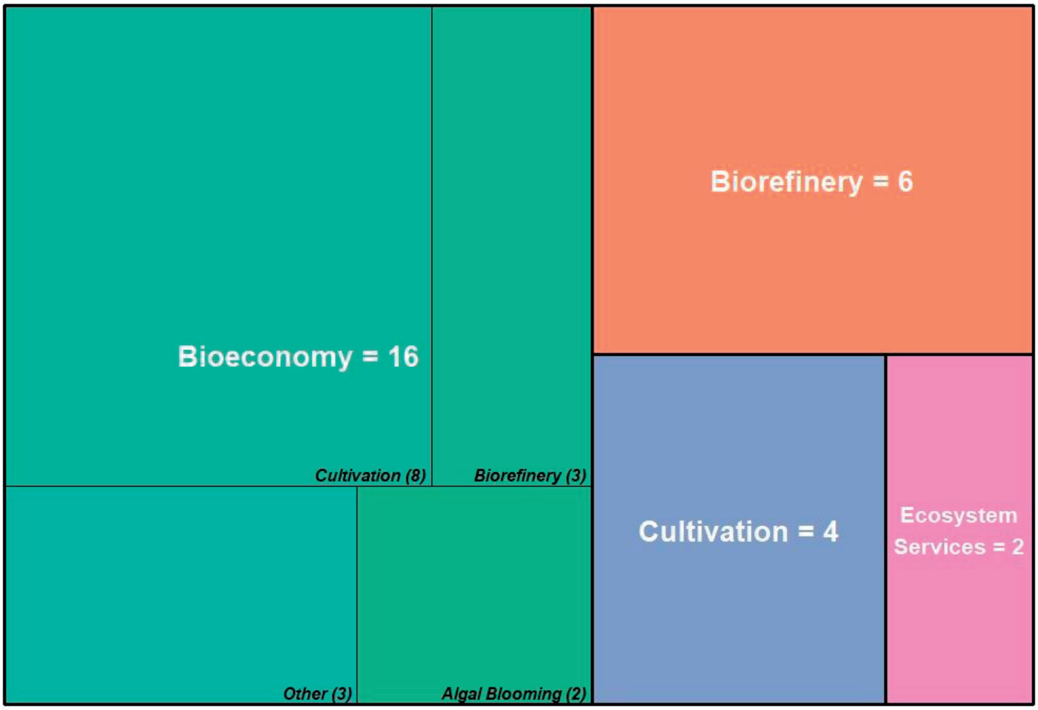

Figure 5. Primary topics of papers included in the review relating to circular bioeconomy and the underutilised biomasses.

3.4. Application to aquafeeds

In total 62 (n = 62, Figure 3) papers passed through the eligibility selection relating to biomasses and derived proteins from underutilised blue and green production systems in the aquafeed industry. There was a large majority of papers relating to seaweed in the aquafeed industry, with many publications (n = 52) far outnumbering those for perennial grasses (n = 7) and perennial legumes (n = 3). Literature investigating seaweeds could be divided into three different categories, studies of green seaweeds, red seaweeds and brown seaweeds. There was an identical number of publications for green and red seaweeds but notably fewer for brown seaweeds as aquafeed ingredients. *Ulva* sp., *Gracilaria* sp. and *Laminaria* sp. were the three most referenced seaweed species in this review. There was a clear split in the literature relating to the application of underutilised biomasses either as a direct dietary protein ingredient (54%) or as a supplemented feed additive (46%). Feed additive research referred to these sources as potential antioxidants or immune-stimulating ingredients. There was a range of different aquaculture species that were featured in the research articles, the most common of which were teleost fish (Figure 6) where a wide range of different species had been investigated in the existing literature. Most of these fish species were herbivore or omnivores, such as Nile Tilapia (*Oreochromis niloticus*). However, some carnivorous species which have a higher dietary protein demand appeared in a small number of papers, most notably, Atlantic salmon (*Salmo salar*) and Rainbow trout (*Oncorhynchus mykiss*). Many papers (n = 7) referred to popular aquaculture shrimp species such as Whiteleg shrimp (*Litopenaeus vannamei*). In the case of other aquaculture species, the dietary requirements of these species are such that the novel proteins and biomasses tested could be used as complete diets rather than just a dietary component. The unspecified papers represent those papers that discuss the potential of different “new materials” in aquafeeds in general with experimental testing.

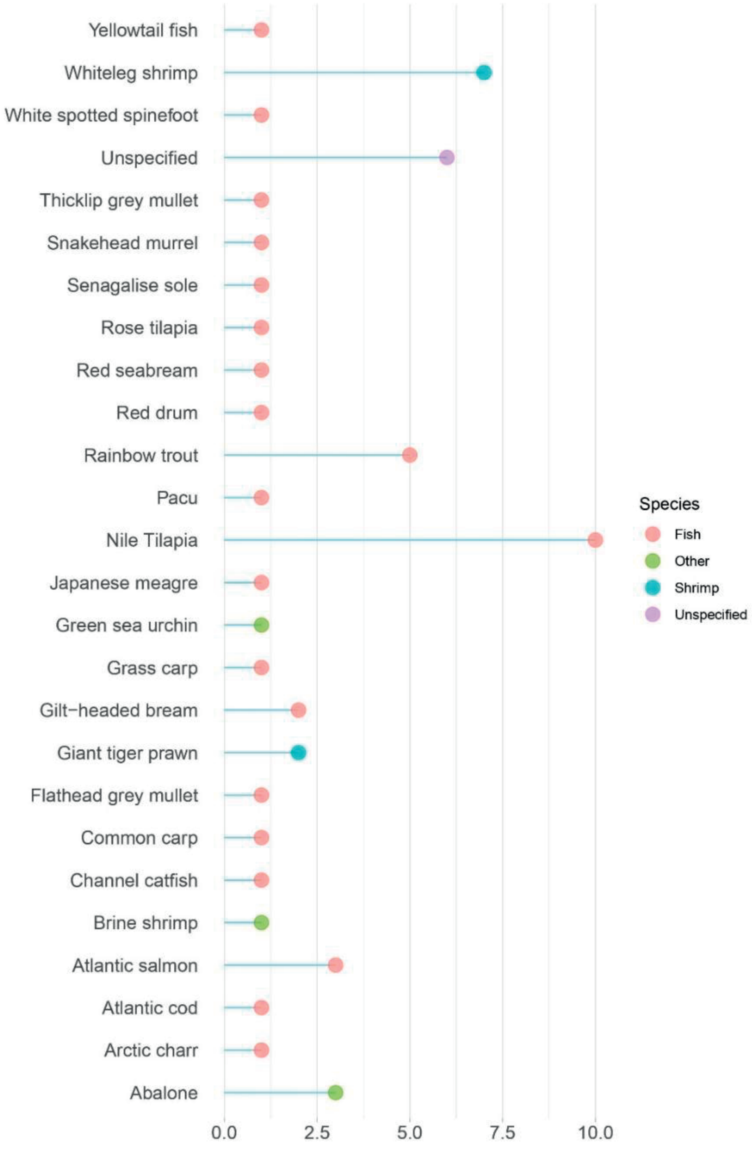

Figure 6. Aquaculture species featured in the papers included in the review relating to aquafeeds and the underutilised biomasses. The unspecified category refers to those papers referring to the biomasses and their general potential for aquafeed inclusion without a species identified.

4. Discussion

This review aimed to map the ecosystem services provided by the underutilised production systems of seaweed, perennial grasses, and legumes, to assess how the biomasses and proteins derived from these systems are currently incorporated into the circular bioeconomy research and how they are currently incorporated into the aquafeed industry. The findings of the review are discussed below and were used to create a circular bioeconomy model that connects the selected biomasses to aquaculture and highlight the research needs to successfully implement such a system.

A broad variety of quantitatively reported ES was found in the reviewed literature, clearly stating the cultural, supportive, provisional and regulating importance of seaweed, perennial grass and legume ecosystems for environmental health, as the main influencing factor for human wellbeing. Seghetta et al., (2016b), for instance, described seaweed cultivation as engineered ecosystem services, facilitating a regenerative circular bioeconomy and suitable for fish feed models. Provisioning services of seaweed as food, bioenergy feedstock, and raw materials were in focus in most reviewed papers. We hence hypothesize that these are main drivers for the large-scale cultivation in parts of the world without explicit heritage relation to seaweed for human consumption (Augyte et al., 2017; Coleman and Wernberg 2017). This can be depicted by the increase of production rates from 1000 t yr^{-1} in the 1970s to 30.1 million t in 2016 (Hurtado et al., 2019; de Queiroz Andrade et al., 2020). Grassland ecosystems on mineral soils are globally one of the most extensive habitat types, accounting for 20-40% of the total earth's land area, but 69% of the world's productive agricultural area (Scordia and Cosentino 2019). Notably for grasslands, the provision of ES is highly dependent on general ecosystem health and intensification. We find that this has been in particular stated in connection with no-till cultivations as compared to grassland under crop-rotation (Castelli et al., 2017; Scordia and Cosentino 2019; Iepema et al., 2020) that did not only provide higher N-use efficiencies, reducing leaching or run-off with surface waters, but also resulted in higher crude protein contents of the biomass, a highly relevant factor for green protein biorefining. Peatlands drained for agriculture only cover 0.3 % of the total land area, but release about 5–6 % of the total anthropogenic GHG (Joosten 2016). In contrast, paludiculture on rewetted peat soils for the production of biomass for protein extraction has the potential to reduce GHG by significant amounts as compared to a drained status-quo (e.g. Günther et al., (2020)), therewith depicting the importance of carbon sequestration and climate regulation as ES from these ecosystems.

Our results highlighted that ES and perennial grass systems, either on mineral or wet peat soils, are going hand in hand for a contribution towards the sustainable terrestrial production of protein for a variety of purposes (Humphreys et al., 2013; Chen et al., 2016; Elgersma and Søegaard 2016; Everson and Everson 2016). However, more research on optimal biomass quality characteristics for protein biorefinery and upscaling is needed. Future considerations for the establishment of sustainable agri-, and aquacultural systems for protein production should consider the variety of ES evaluated within the literature review. As Zhang and Thomsen (2019) proposed in an example of seaweed cultivation is an ecosystem-based management framework between industry, local authorities, and research crucial for a successful “sustainable development of a (macroalgal) bioeconomy that contributes to the restoration of the balance of Earth’s biogeochemical cycles”.

Few articles, compared to the other categories in this review, regarding under-utilised green and blue biomasses address circular economy and/or bioeconomy, which is also reflected by economy terms not being visible in the word cloud. However, the interest is increasing, indicated by the main part of the related articles being published within the last five years. The opportunities for underutilised biomasses to be incorporated into a circular/bioeconomic model were mainly ascribed to the high biomass productivity and ES performed by the cultivation systems, with circularity mentioned for example upon nutrient cycling (Golberg et al., 2019; Torres et al., 2019; Albrecht and Lukkarinen 2020; Ben Fradj et al., 2020; Saldarriaga-Hernandez et al., 2020; Winkler et al., 2020). These opportunities are highly researched, whereas the lower amount of literature on biorefinery applications, highlights a key challenge. Even though research interest is increasing in the field of biorefinery of these biomasses, development is still needed, especially on up-scaling of processing methods. Moreover, a knowledge gap in the case of biorefinery products, such as protein, were found regarding appearance in bioeconomy research. However, based on the reviewed literature, the underutilised green and blue biomasses were found to have the potential to support a circular bioeconomic model. Nonetheless, more research and development are needed for efficient utilisation, especially for biorefined products, such as protein extracts, from the biomasses. However, beyond the articles included in this review, several articles are to be found on protein extraction from seaweed and perennials, looking into different extraction methods including biorefinery cascades. These were not captured in the search of this review, as they do not contain any of the economy terms used in the search string. Further, neither of the articles included in this review touching upon biorefinery cascades with a circular economy approach includes an economic analysis, reflecting a general lack in the literature connecting economy and these novel protein production systems. With the still growing interest in green/blue biorefineries, seen with the increased number of published articles in this field, it must only be a matter of time before the technological challenges will be improved, and business cases are to be analysed.

Besides the technological challenges, the literature calls for a political framework to navigate within for these underutilised biomasses. There is indeed high political motivation to increase the green and blue bioeconomy, e.g. the European Commission has announced that “European Bioeconomy needs to have sustainability and circularity at its heart” (European Commission 2018). The European Commission has set up a Bioeconomic Strategy with three key aspects being: 1) developing new technologies and processes for the bioeconomy, 2) developing markets and competitiveness in bioeconomy sectors, and 3) pushing policymakers and stakeholders to work more closely together (European Commission 2017b). However, the lack of a political framework was the most mentioned challenge by the articles included in this review, suggesting that concrete frameworks must be laid out to support bioeconomy in the novel protein production sector. Lewandowski et al. (2016) suggest that “Remuneration for non-market ecosystem services should include funding for particularly high-service provision, e.g., flood risk reduction, soil protection, nitrate mitigation etc.” to recognize ecological benefits of these biomasses (Lewandowski et al. 2016).

This review revealed substantial interest in the aquafeed industry to find novel alternatives to traditional aquafeed proteins. The literature that was retrieved by this paper´s search string revealed a strong dominance in the existing literature for exploration of the potential of seaweed biomasses and novel green proteins from seaweed in the diets of both cultured fish and shrimp species as well as other species such as the mollusc, abalone. This pattern simultaneously highlighted a huge gap in the aquafeed research literature. There is a wide variety of cultivated perennial grasses and perennial legumes, such as Miscanthus (*Miscanthus giganteus*), Switchgrass (*Panicun virgatum*), Reed canary grass (*Phalaris arundinacea*), Red clover (*Trifolium pratense*) and White clover (*Trifolium repens*) (Muylle et al., 2015; Scordia and Cosentino 2019). Despite these sources being listed as being of high energy value, and underutilised in the wider literature, no research was returned in the search of this review for any of these biomasses in aquafeed industry. This positive publication bias toward seaweed is not surprising, since seaweeds represent a natural dietary component for many herbivorous and omnivorous aquaculture species making it an obvious target for raw material exploration of many seaweed species (Angell et al., 2016; Wan et al., 2019). This also partially explains why there was a greater number of publications on non-carnivorous aquaculture species captured in this review. Research attention has favoured alternative protein sources for aquaculture that are not dependent on terrestrial agriculture due to limited land space, high water requirements, and the application of fertilizers (Godfray et al., 2010). Soybean, a monocultured annual legume currently dominates the feed market for many monogastric species including fish and faces high market competition for human food products (Ytrestøyl et al., 2015). This dominance puts increasing environmental pressure on the soy production systems and drives up the price of this material creating an impetus to use other terrestrial biomasses that are available to the market. It will therefore be important to give greater research focus to the potential of underutilised perennial grasses and legumes in aquaculture.

The dominance of fish species related papers found by this review reflects the dominance of these species in the aquaculture industry in general (Cai et al., 2019). The bias towards herbivorous and omnivorous fish species especially, reflects the more flexible nature of the dietary requirements of those fish compared with carnivorous species like many salmonids that have higher dietary protein needs and are more sensitive to the vegetable and plant consumption (National Research Council 2011; Booman et al., 2018). The review revealed the popularity of using tilapia and carp as a test organism for the selected biomasses. The success of different seaweeds as fish meal replacements for Nile Tilapia (*Oreochromis niloticus*) aquafeeds seemed to be highly species dependent. For example, at 10% inclusion levels, the seaweeds *Porphyra dioica* (Rhodophyta) and *Ulva* spp. (Chlorophyta) did not negatively impact growth performance and showed potential for inclusion, whereas *Gracilaria vermiculophylla* (Rhodophyta) negatively affected growth (Silva et al., 2015). In all cases in the same study, the seaweeds altered the digestive morphology of the fish, something that needs to be carefully considered for gut health and welfare especially for carnivorous species, which may be more sensitive. Silva et al., (2015) suggest seaweed inclusion should be considered on a case-by-case basis as not all seaweeds will have the same potential for aquafeeds, so equal attention should be paid to exploring Chlorophyta, Rhodophyta and Phaeophyta. Future research should evaluate the use of seaweeds, perennial grasses, and legumes in the diets of a wider range of aquaculture species and in a balance of herbivores, omnivores and carnivores reflecting the needs of the industry. For reviewed studies with carnivorous species like salmonids, the biomasses were investigated purely for their roles as feed additives and not as protein replacements compared with other species which equally explored protein replacement potential. These studies showed that supplementation with low levels of seaweeds provided positive benefits such as increased feed intake, protein efficiency and immune responses (Palstra et al., 2018; Kamunde et al., 2019). It will be important in future studies to consider both applications of these underutilised biomasses as a protein replacement and as feed additives - both roles that not only will bring production, environmental and economic benefits to the aquafeed industry, but also the processing necessary to optimise these benefits.

There is additional opportunity to utilise seaweeds, perennial grasses and legumes indirectly for aquafeed protein sources for example using the biomass as a substrate for other protein sources such as for rearing insects or growing single celled proteins (Rajoka 2005; Tinder et al., 2017; Sharma et al., 2018; Swinscoe et al., 2020). This could be particularly beneficial for aquaculture species with higher protein levels than seaweed can provide, such as Atlantic salmon (*Salmo salar*) (Angell et al., 2016). Similar models are being investigated in the existing research such as lignocellulosic biomasses from wood to culture yeast proteins (Øverland and Skrede 2017; Sharma et al., 2018). Perennial grasses and legumes are an untapped source of lignocellulosic biomass which would benefit from further investigation for single cell protein production for the aquafeed industry (Warnke and Ruhland 2016; Scordia and Cosentino 2019). Seaweeds have been used to rear Black soldier fly larvae and produce yeast (Sharma et al., 2018; Swinscoe et al., 2020) for aquaculture and offer a promising option to exploit these under-utilised biomasses effectively, yet research questions remain in regard to upscaling these processes, their economic competitiveness and safety for example from heavy metal transfer from seaweeds across trophic levels (Biancarosa et al., 2018; Sharma et al., 2018).

This review revealed the high-level potential and the need to develop a circular bioeconomy for both: the utilisation of raw materials and equally for the aquafeed industry in light of environmental and economic sustainability. However, there are substantial research gaps on how to establish and implement such circular models (Kusumowardani and Tjahjono 2020; Venkata Mohan et al., 2020). We present a model based on the key findings of this review as an example of a circular bioeconomy that connects seaweed, perennial grasses, and legumes to the aquafeed industry (Figure 7). This model has the potential to 1) address the market need for the aquaculture industry, 2) make the use of resources more efficient and 3) to boost ecosystem services at the same time. The biomasses and production systems are at the core of the circular bioeconomy models (Venkata Mohan et al., 2020) and have inherent value to boost supporting and regulating ecosystem services, such as nutrient retention and carbon sequestration. They are a source of additional value for processing and biorefinery to extract high quality protein as well as other nutritionally valuable compounds for the aquafeed industry (Seghetta et al., 2016a) or even as substrates to produce other high value proteins. This will support more sustainable aquaculture, and facilitate future food security, a provisioning service. To close the loop, side streams such as fish waste or nutrient rich outflow water could act as fertilizer to enhance production and the associated ecosystem services, such as in integrated multitrophic aquaculture (IMTA), having the additional benefit of reducing the environmental impact of aquaculture (Silva et al., 2015). To implement such a model, we identified several key research needs: research into the effective management of these biomass systems, optimizing and upscaling biorefinery processes, optimizing dietary application and formulation of aquafeeds containing these resulting protein sources and investigating efficient and safe ways to use aquaculture side streams as crop fertilizer. It will also be essential that legislation and political incentive support the development of such promising economies.

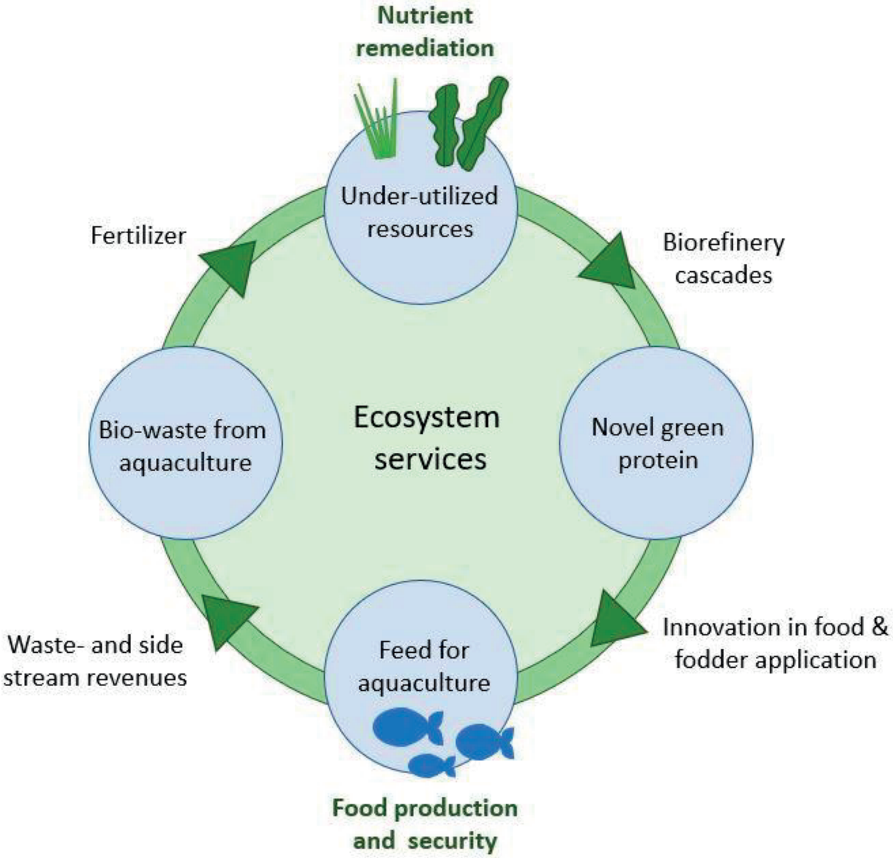

Figure 7. Circular bioeconomy model developed from the findings of this review to highlight the potential for cross-discipline synergy, and opportunities to connect the underutilised biomasses, seaweed, perennial grasses, and perennial legumes efficiently and sustainably to the aquafeed industry.

5. Conclusion

Seaweeds, perennial grasses, and legumes are underutilised biomasses that, if well managed, have the potential to significantly boost ecosystem services, most notably climate and nutrient regulation. At the same time, these biomasses are untapped resources suitable for processing and inclusion in aquaculture feeds, a valuable and much sought-after provisioning service. While much of the research agrees that a circular bioeconomy is well-suited to both aquafeed production and for utilising biomass, we identified a major research gap on how this might be achieved for either sector. While there is consent that optimizing biorefinery systems is crucial, we highlighted a lack of production system evaluation regarding economically and environmentally sustainable utilisation of the biomasses. Moreover, we found the request for political framework, which might help connecting the dots through the valorising chain taking advantage of the ES. This review presented a model for how the selected underutilised biomasses might be connected in a circular bioeconomy to the aquafeed industry. In conclusion, we found that to achieve such a desirable model, more research is needed into 1) optimizing biorefinery processes, 2) application of derived products to the aquafeed industry, and 3) efficient application of aquaculture side streams to close the loop and in-turn support production of seaweed, perennial grass, and perennial legume systems.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

All three corresponding authors contributed substantially and equally to the conception, methodology, outline, and writing of the manuscript and are co-first authors. All authors listed contributed to the writing and reading of the manuscript and approved the final manuscript.

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Data Availability Statement

Datasets generated and analysed for this study are available on request to the corresponding authors.

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Paper IV

Supplementary material

Supplementary Material

1 Keywords and Search Strings

1.1 Scopus

TITLE-ABS-KEY (("grass* protein*" OR "*alg* protein*" OR "seaweed* protein*" OR phalaris OR typha OR "crude protein" OR phragmites OR "grass* meal*" OR "*alg* meal*" OR "seaweed* meal*" OR "paludicult*" OR peatland* OR lolium OR "tall fescue" OR festuca* OR "reed canary grass" OR *clover* OR alfalfa OR "grass* land*" OR "seaweed* cult*" OR "seaweed* farm*" OR "*alg* cult*" OR "*alg* farm*" OR "seagricult" OR "grass* bioref*" OR "seaweed* bioref*" OR "*alg* bioref*" OR ulva OR saccharina OR porphyra OR gracilaria OR laminaria OR ascophyllum OR "kikuyu grass" OR sargassum OR palmaria OR alaria OR undaria OR fucus) AND ("bioecono*" OR "circular *econo*" OR "green *econo*" OR "sustainable *econo*") OR ("ecosystem service*" OR "natural capital*" OR "nature's benefit to people") OR ("aquaculture food*" OR "aquaculture feed*" OR "fish feed*" OR aquafeed* OR "farm* fish feed*" OR "fish meal replacement" OR "aquacult* protein" OR "tilapia feed*" OR "salmon* feed*" OR "fish fod*" OR "grass* protein* in aquacult*" OR "seaweed protein* aquacult*" OR "fish meal sub*" OR "grass meal*" OR "seaweed meal*") AND NOT (micro* OR "human*" OR insect* OR genetic* OR pea OR soy* OR poultry*))

1.2 Web of Science

TS=(("grass* protein*" OR "*alg* protein*" OR "seaweed* protein*" OR phalaris OR typha OR "crude protein" OR phragmites OR "grass* meal*" OR "*alg* meal*" OR "seaweed* meal*" OR "paludicult*" OR peatland* OR lolium OR "tall fescue" OR festuca* OR "reed canary grass" OR *clover* OR alfalfa OR "grass* land*" OR "seaweed* cult*" OR "seaweed* farm*" OR "*alg* cult*" OR "*alg* farm*" OR "seagricult" OR "grass* bioref*" OR "seaweed* bioref*" OR "*alg* bioref*" OR ulva OR saccharina OR porphyra OR gracilaria OR laminaria OR ascophyllum OR "kikuyu grass" OR sargassum OR palmaria OR alaria OR undaria OR fucus) AND (("bioecono*" OR "circular *econo*"

OR "green *econo*" OR "sustainable *econo*") OR ("ecosystem service*" OR "natural capital*" OR "nature's benefit to people") OR ("aquaculture food*" OR "aquaculture feed*" OR "fish feed*" OR aquafeed* OR "farm* fish feed*" OR "fish meal replacement" OR "aquacult* protein" OR "tilapia feed*" OR "salmon* feed*" OR "fish fod*" OR "grass* protein* in aquacult*" OR "seaweed protein* aquacult*" OR "fish meal sub*" OR "grass meal*" OR "seaweed meal*")) NOT (micro* OR "human*" OR insect* OR genetic* OR pea OR soy* OR poultry*))

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