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Shaping the salmonid gut microbiota

Meta-analytical insights and dietary approaches to enhance fish health and performance

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Shaping the salmonid gut microbiota. Meta-analytical insights and dietary approaches to enhance fish health and performance

Abstract

Aquaculture is the fastest-growing food production sector globally and plays a crucial role in ensuring future food security. However, sustainable intensification of aquaculture requires novel feed ingredients and strategies that promote fish health and performance while reducing dependence on conventional protein sources such as fishmeal and soy. The gut microbiota represents a key biological interface between diet, host physiology, and environmental factors, profoundly influencing nutrient utilization, immune function, and overall fish wellbeing. This thesis examined the influence of biotic, abiotic, and particularly dietary factors on the intestinal microbiota of salmonid species, with a specific focus on rainbow trout (*Oncorhynchus mykiss*), and the potential of microbial-based feed ingredients to support sustainable aquaculture.

The first study applied a meta-analytical approach to integrate data from published sequencing studies on Atlantic salmon and rainbow trout. The analysis revealed that the gut microbiota composition of salmonids is strongly influenced by methodological, environmental, and host-related factors, underscoring the need for standardization in sampling and sequencing protocols.

The second study evaluated four filamentous fungi, *Aspergillus oryzae*, *Neurospora intermedia*, *Rhizopus delemar*, and *Rhizopus oryzae*, grown on ethanol-production stillage as alternative protein sources in rainbow trout diets. While these fungal biomasses exhibited comparable but slightly lower digestibility scores compared with the control, their inclusion altered intestinal microbial diversity, highlighting their potential as sustainable feed ingredients pending improvements in digestibility.

The third study assessed the effects of dietary supplementation with two yeast probiotics, *Kluyveromyces marxianus* and *Rhodosporidium babjevae*, on rainbow trout gut microbiota, immune-related gene expression, and growth performance. Supplementation with *R. babjevae* modulated the abundance of beneficial bacterial taxa and influenced immune-related gene expression, supporting its candidacy as a promising probiotic for aquaculture feeds.

Collectively, this work advances the understanding of how feed composition and microbial inputs affect the gut ecosystem of farmed salmonids. The findings contribute to the development of more sustainable and health-promoting aquafeed formulations, facilitating the transition toward environmentally responsible aquaculture practices.

Keywords: 16S rRNA gene sequencing, fresh water, gut bacteria, microbiome, salmonid, filamentous fungi, yeast probiotic, nutrient digestibility, immunity

Formandet av laxfiskens tarmmikrobiota – meta-analytiska insikter och näringsstrategier för att stärka fiskhälsa och prestation

Abstract

Akvakultur är den snabbast växande livsmedelssektorn globalt och spelar en central roll för framtida livsmedelssäkerhet. En hållbar intensifiering kräver dock nya foderingredienser och strategier som främjar fiskhälsa och prestation, samtidigt som beroendet av fiskmjöl och soja minskas. Tarmmikrobiotan utgör ett viktigt biologiskt gränssnitt mellan diet, värdfysiologi och miljö, och påverkar näringsupptag, immunfunktion och fiskens välbefinnande. Denna avhandling undersökte hur biotiska, abiotiska och särskilt dietära faktorer påverkar tarmmikrobiotan hos laxfiskar, med fokus på regnbåge (*Oncorhynchus mykiss*), samt potentialen hos mikrobiellt baserade foderingredienser för en mer hållbar akvakultur.

Den första studien använde en meta-analytisk metod för att integrera sekvensdata från atlantlax och regnbåge. Resultaten visade att tarmmikrobiotans sammansättning starkt påverkas av metodologiska, miljömässiga och värdrelaterade faktorer, vilket understryker behovet av standardisering i provtagning och sekvensering.

Den andra studien utvärderade fyra filamentösa svampar, *Aspergillus oryzae*, *Neurospora intermedia*, *Rhizopus delemar* och *Rhizopus oryzae*, odlade på restprodukter från etanolproduktion som alternativa proteinkällor i regnbågsfoder. Trots något lägre smältbarhet än kontrollen förändrade svamptillskotten tarmens mikrobiella mångfald, vilket visar deras potential som hållbara foderingredienser med förbättrad smältbarhet.

Den tredje studien analyserade effekter av två jästprobiotika, *Kluyveromyces marxianus* och *Rhodosporidium babjevae*, på regnbågens tarmmikrobiota, immunrelaterade gener och tillväxt. *R. babjevae* gynnade nyttiga bakterier och påverkade immunrelaterade genuttryck, vilket stödjer dess potential som probiotikum i fiskfoder.

Sammantaget bidrar arbetet till ökad förståelse för hur fodersammansättning och mikrobiella tillskott formar tarmekosystemet hos odlade laxfiskar. Resultaten stöder utvecklingen av mer hållbara och hälsofrämjande fodersammansättningar för en miljömässigt ansvarsfull akvakultur.

Nyckelord: 16S rDNA, sötvatten, tarmmikrober, mikrobiom, laxfiskar, filamentösa svampar, jästprobiotika, näringsupptag, immunförsvar

Dedication

To my beloved mother, for supporting every choice I have made.

道阻且长，行则将至。
Though the road is long and filled with trials, every step forward brings you closer to your destination.

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Cao, S., Dicksved, J., Lundh, T., Vidakovic, A., Norouzitallab, P., & Huyben, D. (2024). A meta-analysis revealing the technical, environmental, and host-associated factors that shape the gut microbiota of Atlantic salmon and rainbow trout. Reviews in Aquaculture, 16(4), 1603–1620. https://doi.org/10.1111/RAQ.12913

II. Cao, S., Dicksved, J., Carlberg H., Langeland M., Karimi S., Taherzadeh M. J., & Vidakovic A. Dietary filamentous fungi modulate microbial composition in the intestine of rainbow trout (*Oncorhynchus mykiss*). (Manuscript)

III. Cao, S., Dicksved, J., Lundh, T., Vidakovic, A., Huyben, D., Volkmar P., Blomqvist J., & Norouzitallab, P. Supplementation of probiotic yeast (*Rhodosporidium babjevae*) enhances gut microbiota, intestinal barrier integrity and immune function in rainbow trout (*Oncorhynchus mykiss*), while *Kluyveromyces marxianus* did not. (Manuscript)

Paper I is published open access.

The contribution of Shuowen Cao to the papers included in this thesis was as follows:

I. Planned the meta-analysis, carried out systematic literature search, collected data, processed datasets, performed the bioinformatic and statistical analyses, and wrote the manuscript.

II. Conducted sampling, analysed gut microbiota and growth performance, performed statistical analyses, and wrote the manuscript.

III. Planned the experiment and partly carried out feed preparation, carried out feeding and collection of feed waste, collected gut contents, intestine, and feed samples, analysed gut and feed microbiota, carried out qPCR, analysed gene expression, performed the statistical analyses, and wrote the manuscript.

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Abbreviations

GIT	Gastrointestinal tract
PRR	Pattern recognition receptor
TLR	Toll-like receptor
MAMP	Microbial-associated molecular pattern
PAMP	Pathogen-associated molecular pattern receptor
WG	Weight gain
SGR	Specific growth rate
VSI	Viscero-somatic Index
HSI	Hepato-somatic Index
FCR	Feed conversion ratio
ADC	Apparent digestibility coefficient
NMDS	Non-metric multidimensional scaling
PCoA	Principal coordinates analysis
LEfSe	Linear discriminant analysis effect size
LDA	Linear discriminant analysis
ANOVA	Analysis of variance
PERMANOVA	Permutational multivariate analysis of variance
ANOSIM	Analysis of similarities

1. Introduction

1.1 Aquaculture overview

Global fisheries and aquaculture production reached a record with 223.2 million tonnes in 2022, consisting of 185.4 million tonnes of aquatic animals worth USD $452 billion and 37.8 million tonnes of algae (FAO, 2024). For the first time, farming of aquatic animals surpassed capture fisheries with an estimated production of 94 million tonnes worth USD $296 billion, representing 51% of the total production (FAO, 2024). In 2022, marine areas contributed 115 million tonnes, or 62% of total production, of which 69% came from capture fisheries and 31% from aquaculture (FAO, 2024). Inland waters produced 70 million tonnes, representing 38% of the global total, with aquaculture accounting for 84% and capture fisheries for 16% (Figure 1). Global capture fisheries production has remained stable since the late 1980s, constrained by the full or excessive exploitation of many wild stocks. In contrast, aquaculture has expanded rapidly over the same period, with global production increasing by 3.7% between 2000 and 2023 (FAO, 2024).

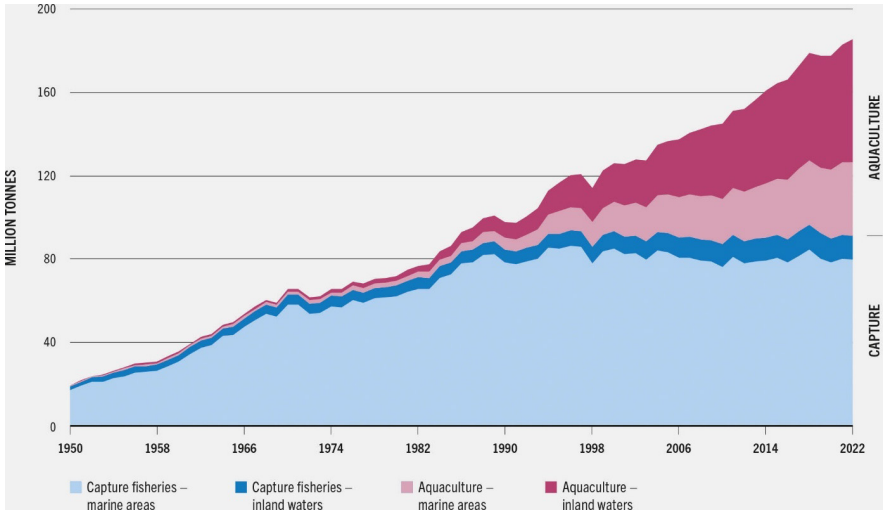


Figure 1. Global fisheries and aquaculture production of aquatic animals (FAO, 2024).

In Europe, Norway is the world's sixth-largest producer of aquaculture fish. In 2023, Norwegian aquaculture production reached 1.6 million tonnes of aquatic organisms, valued at EUR €10.0 billion, surpassing the combined production of the entire European Union (EU). Norwegian aquaculture production is dominated by Atlantic salmon, complemented by substantial production of large trout (>1.2 kg) (FEAP secretariat, 2025). Within the EU, aquaculture has expanded considerably as a key component of the blue economy, attaining 1.1 million tonnes in volume and EUR €4.8 billion in value in 2022, although capture fisheries continue to represent the principal source of aquatic animal production (Eurostat, 2025). Spain was responsible for about 23.1% of the EU's total production of farmed aquatic organisms in 2023, followed by France (17.8%), Greece (13.4%), and Italy (12.3%) (Eurostat, 2025). Finfish species, particularly trout, seabream, seabass, carp, tuna, and salmon, together with molluscs, primarily mussels, oysters, and clams, comprised nearly all EU aquaculture production by weight in 2023 (Eurostat, 2025). Among these, rainbow trout (*Oncorhynchus mykiss*) represented the most valuable farmed species, accounting for 17.7% of total aquaculture production value, followed by seabass, seabream, and oysters.

In Sweden, total sea-fisheries landings reached approximately 143,000 tonnes (live weight) in 2023, representing a 3% increase compared to 2022, with a total value of SEK 1027 million (SCB, 2024). In contrast, aquaculture production remains relatively limited, amounting to 9700 tonnes of fish for consumption in 2023, of which rainbow trout accounted for approximately 87% (Jordbruksverket, 2024; SCB, 2024). By 2024, aquaculture output had declined slightly to 9100 tonnes (Jordbruksverket, 2025). The Swedish aquaculture sector is characterized by a narrow species range, modest scale, and slow growth (Barquet et al., 2023). Capture fisheries continue to dominate national aquatic production, particularly through landings of herring and sprat from the Baltic Sea, while aquaculture remains a small but stable component of Sweden's blue economy (Jordbruksverket, 2025; SCB, 2024).

1.2 Aquafeed industry

Fish are a nutritious source of high-quality protein, fat, minerals and vitamins that have beneficial effects on human health (Ahmed et al., 2022; Dale et al., 2019). The demand for increased fish production and consumption has been

growing worldwide and is now at an all-time high of 21 kg/capita (FAO, 2024). As a result, aquaculture has been expanding to meet this demand, although a growing aquaculture industry requires more aquafeed production. In conventional aquafeed production, fishmeal and fish oil, have long been considered the gold standard protein and lipid source for carnivorous, species such as salmonids and shrimp, due to the ideal amino acid profile, high digestibility, and inclusion of essential omega-3 fatty acids (FAO, 2024). To avoid conflicts with human consumption, aquafeed utilizes the remnants from wild fisheries. However, the supply is inherently limited by the constraints on wild fisheries, which are increasingly affected by harvest quotas, climate variability, and sustainability concerns. As aquaculture grows, the demand for fishmeal and fish oil far outpaces what can be produced, leading to high prices and consequently more expensive aquafeeds and less fish farm profitability. Thus, seeking alternative feed sources to replace fishmeal and fish oil is greatly needed to support the growth of the aquaculture industry and production of seafood to feed a growing human population.

Salmonids need high-protein, high-lipid, low-carbohydrate diets, that include essential amino acids, omega-3 fatty acids, vitamins, minerals, and sometimes functional additives for optimal growth (Jobling, 2016; National Research Council, 2011). Due to the large production, moderate-protein and high-lipid plants, such as soybeans, corn, and rapeseed, naturally became early candidates to replace fishmeal and fish oil in salmonid aquafeeds (Ringø et al., 2006; Sallam et al., 2021). However, plants contain anti-nutritional factors, less protein, and dissimilar amino acid profile compared to fishmeal, and therefore results in diverse problems such as essential amino acid deficiencies, lower digestibility, reduced feed intake, suppressed immune response, and even intestinal inflammation (Hussain et al., 2024; Lin & Cheng, 2017), which further results in reduced fish production and economic losses. Processing plants into protein concentrates and isolates can increase protein content and digestibility while avoiding the inhibitory compounds (Chen et al., 2024; Egerton et al., 2020; Han et al., 2022; Mugwanya et al., 2023), but with extra costs for the production system. Due to sustainability issues, the inclusion of plant-based proteins and oils have been increasing during the last decades to replace fish meal and fish oils (Figure 2). Plant-based protein and lipid sources have partially alleviated the dependence on fishmeal and fish oil in aquafeed production. However, the

expanding biofuel and biogas industries are generating new competing demands for these resources (Jameel et al., 2024; Voloshin et al., 2016). Therefore, there is an urgent need to identify alternative protein and lipid sources, along with suitable dietary supplements, that can sustain fish growth and health while reducing reliance on conventional feed ingredients.

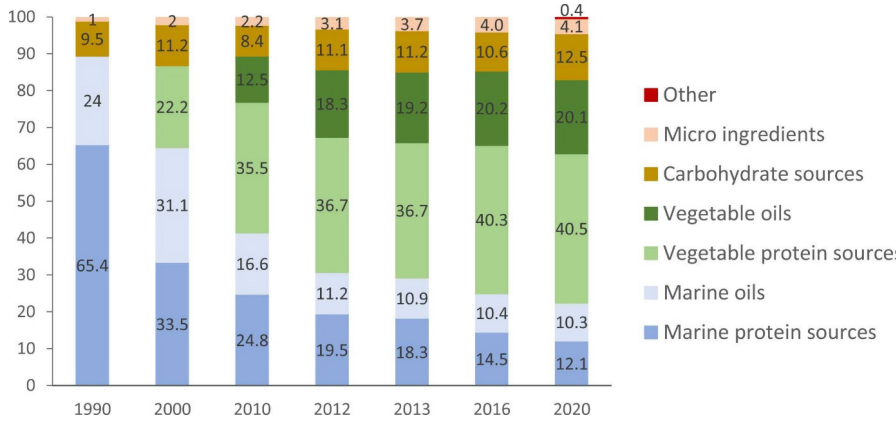


Figure 2. Sources of feed ingredients (% of feed) in Norwegian salmon feed between 1990 and 2020 (Aas et al., 2022).

1.3 Alternative aquafeed sources

To address the limitations of plant-based diets, novel ingredients, including insect meals, single-cell proteins from microalgae, fungi, and bacteria, as well as rendered by-products from terrestrial animals, have been tested as aquafeed sources (Serra et al., 2024). Insect meals, particularly those from black soldier fly (*Hermetia illucens*) larvae, have gained considerable attention due to their high protein content, favourable amino acid profile, low carbon footprint and efficient production using organic waste streams (Fantatto et al., 2024; Huyben et al., 2019; Rimoldi et al., 2019; Terova et al., 2021). Single cell proteins and single-cell oils, derived from microorganisms such as microalgae, fungi, and bacteria, represent another promising category, with microalgae being especially valuable for their ability to supply both protein and essential long-chain omega-3 fatty acids (Amara & El-Baky, 2023; Chamodi et al., 2025; Glencross et al., 2020; Vasilaki et al., 2023). Additionally, rendered by-products from terrestrial animals, including poultry by-product meal and blood meal, serve as cost-

effective protein sources (Beketov et al., 2020; Wang et al., 2023). However, their use is often limited by consumer perception and regulatory constraints (Shurson et al., 2023). Collectively, these emerging alternatives offer complementary strategies to reduce dependence on marine-derived ingredients while supporting the growth and sustainability of aquaculture.

1.3.1 Filamentous fungi

Filamentous fungi as protein sources are drawing attention as sustainable and high-quality protein sources in aquaculture and an alternative to fishmeal. Their biomass is rich in crude protein (30-60%), essential amino acids, vitamins, and bioactive compounds, which support growth and nutritional requirements in fish (Karimi et al., 2023). Certain species, such as *Fusarium venenatum* and *Trichoderma reesei*, can be cultivated on low-cost agro-industrial substrates, producing protein-rich biomass while promoting sustainable feed production (Akinsemolu & Onyeaka, 2025; Aro et al., 2023; Gnaim et al., 2025; Zaki & Said, 2018). In addition to providing essential amino acids, filamentous fungi contain bioactive polysaccharides (β-glucans and chitin), nucleic acids and enzymes that may improve fish gut health, nutrient absorption, and immune responses. For example, a 20% protein replacement by *Paecilomyces variotii* improved feed conversion ratio (FCR) and nutrient utilization efficiency of Atlantic salmon (*Salmo salar*), with an upregulation of several cytokines (Hooft et al., 2024).

In addition to these species, some other filamentous fungi, such as *Aspergillus oryzae*, *Rhizopus oligosporus*, *Rhizopus delemar*, and *Neurospora intermedia*, have also shown good traits as protein sources for salmonids (Gaudhaman et al., 2025; Singh et al., 2021). These fungi are generally recognized as safe (GRAS) for human use and are traditionally used in Asian food fermentations such as miso, soy sauce, amazake, and tempeh (Daba et al., 2021; Karimi et al., 2021; Londoño-Hernández et al., 2017; Rekdal et al., 2024; Rousta et al., 2021; Yang et al., 2021). Their biomass contains approximately 40-55% crude protein (dry weight), with a favourable amino acid profile that supports animal and aquaculture nutrition (Karimi et al., 2021; Pratama et al., 2025). Compared with plant-based proteins, they provide high digestibility and lack many anti-nutritional factors, making them promising alternative feed ingredients (Wang et al., 2023). Moreover, they can be cultivated on diverse agricultural and industrial side streams, enhancing their sustainability and cost-effectiveness (Devanthi

et al., 2024; Uwineza et al., 2021, 2023). Feeding trials in fish have also indicated that partial replacement of fishmeal with their biomass can support growth performance without compromising health (Dawood et al., 2019; Gaudhaman et al., 2025; Ibarruri & Hernández, 2018; Uyar & Uyar, 2023; Singh et al., 2021; Vidakovic et al., 2016).

1.3.2 Dietary yeasts

Yeasts are increasingly recognized as promising alternative protein sources for animal and aquaculture nutrition, owing to their high-quality protein content, balanced amino acid composition, and potential for sustainable large-scale production using industrial side streams (Agboola et al., 2021). A growing body of evidence supports their nutritional efficacy and functional benefits. Singh et al. (2024) investigated the oleaginous yeast *Yarrowia lipolytica* as a feed ingredient for rainbow trout, demonstrating significant improvements in gut microbiota composition, immune modulation, and feed conversion efficiency. Similarly, Huyben et al. (2017) evaluated *Saccharomyces cerevisiae* and *Wickerhamomyces anomalus* in rainbow trout diets, revealing enhanced nutrient digestibility, intestinal morphology, and growth performance. Vidakovic et al. (2016) further showed that supplementation with *S. cerevisiae* supported intestinal integrity and growth in Arctic charr (*Salvelinus alpinus*), underscoring its functional role in maintaining gut health. Moreover, Øverland et al. (2013) demonstrated that yeast-derived protein ingredients, including *Candida utilis*, *Kluyveromyces marxianus*, and *S. cerevisiae*, can effectively replace conventional protein sources in diets for Atlantic salmon without compromising growth or feed utilization efficiency. Collectively, these studies highlight the biotechnological potential of both conventional and non-conventional yeasts as sustainable, functional feed ingredients that contribute to circular and resource-efficient bioeconomy frameworks in animal production systems.

1.4 Gut health

1.4.1 Fish gut health and immune system

The gastrointestinal tract (GIT) of fish plays a fundamental role not only in nutrient digestion and absorption but also in immune regulation and overall

physiological homeostasis (Ray & Ringø, 2014). As the largest mucosal surface exposed to the external environment, the GIT serves as a crucial physical and immunological barrier against pathogens while harbouring a complex and dynamic microbial community that contributes to nutrient metabolism, immune modulation, and disease resistance (Mokhtar et al., 2023). The intestinal epithelium, mucus layer, and associated immune cells constitute an integrated defence network that coordinates both innate and adaptive immune responses (Mokhtar et al., 2023). Pattern recognition receptors (PRRs), including toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors, detect microbial-associated molecular patterns (MAMPs) and activate signalling cascades that regulate cytokine production, inflammation, and antimicrobial peptide synthesis (Mokhtar et al., 2023). A balanced gut microbiota supports epithelial integrity and immune tolerance, whereas dysbiosis can impair barrier function and increase susceptibility to enteric diseases (Klak et al., 2025; Liu et al., 2025). Consequently, dietary interventions, such as probiotics, prebiotics, and functional feed additives including yeast-derived components, have attracted growing interest as strategies to enhance gut health and mucosal immunity by upregulating protective and anti-inflammatory pathways while mitigating excessive pro-inflammatory responses in aquaculture species (Naya-Català et al., 2021; Xiong et al., 2020). Maintaining a stable gut ecosystem is therefore critical for optimizing growth performance, disease resistance, and overall welfare in intensive aquaculture systems.

Fungal-derived antigens, such as β-glucans and other polysaccharides, are well-known immunomodulatory agents that activate the teleost immune system through multiple mechanisms, thereby enhancing protection against pathogens (Figure 3) (Machuca et al., 2022). Following oral administration, β-glucans reach the intestinal lumen, where they are recognized by pathogen-associated molecular pattern receptors (PAMPs), initiating the production of both pro- and anti-inflammatory cytokines and signalling molecules that coordinate innate and adaptive immune responses (Machuca et al., 2022). C-type lectin receptors (CLRs) are key mediators of antifungal immunity, recognizing carbohydrate structures on fungal cell walls and triggering immune processes such as phagocytosis, cytokine secretion, and T-cell activation (Hatinguais et al., 2022). Toll-like receptor 2 (TLR2), another major PRR, detects microbial-associated molecular patterns and activates

downstream signalling pathways leading to the release of pro-inflammatory cytokines, including interleukin-1 beta (IL-1β) and tumour necrosis factor-alpha (TNF-α), which mediate innate immune responses, recruit leukocytes, and facilitate pathogen clearance (Lauriano et al., 2016; Rimoldi et al., 2023; Zheng et al., 2024; Zou & Secombes, 2016). Conversely, transforming growth factor-beta (TGF-β) acts as a critical anti-inflammatory regulator that maintains mucosal immune tolerance and prevents excessive inflammation (Lilleeng et al., 2009; Zhang et al., 2022). Mucin 2 (MUC2) contributes to the mucus layer that traps pathogens and supports microbial equilibrium (Wang, Gao, et al., 2024; Zhu et al., 2024), while occludin (OCLN), a tight junction protein, preserves epithelial barrier integrity and regulates gut permeability (Abdelhafiz et al., 2023; Gaetano et al., 2025). Collectively, these genes and molecular mechanisms illustrate the intricate interplay between immune activation, regulation, and barrier maintenance in the fish intestine, processes that are essential for sustaining gut homeostasis, disease resistance, and overall fish health.

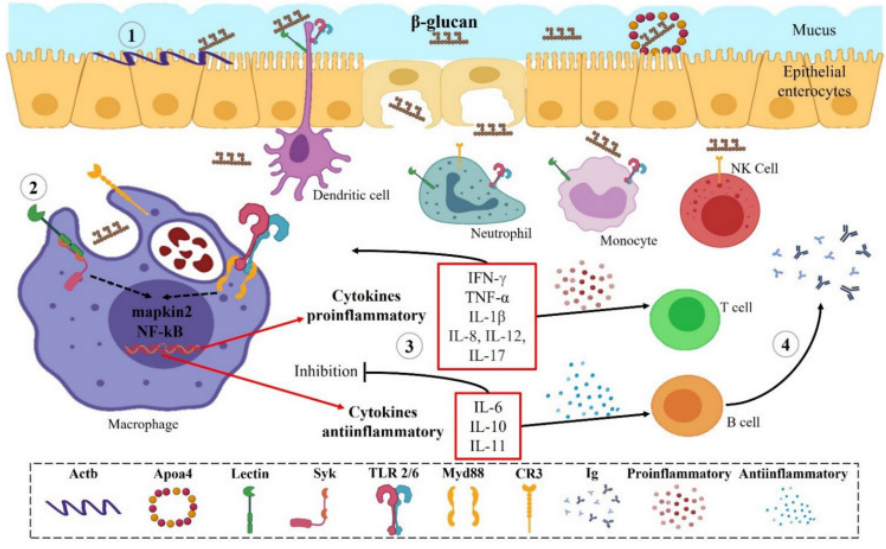


Figure 3. Signalling pathway for yeast β-glucans in teleost fish organisms (Machuca et al., 2022). Illustration of the β-glucan activation pathway in fish. (1) Intestinal enterocytes synthesize metabolic proteins in response to yeast β-glucan and release them into systemic circulation. (2) β-glucan is recognized by PAMP receptors, activating innate immune signalling and NF-κB-mediated gene expression through phosphorylation, ubiquitination, and protein degradation. (3) Resulting pro- and anti-inflammatory cytokines, receptors, and related mediators coordinate and activate adaptive immune responses. (4) B cells activated by β-glucan stimulation produce immunoglobulins.

1.4.2 Gut microbiota and influencing factors

The gut microbiota plays an important role in fish health and performance by mediating nutrient digestion, absorption, and metabolism (Hoque et al., 2023; Luan et al., 2023). It enhances feed utilization by breaking down complex dietary components and producing bioactive metabolites, while simultaneously modulating immune responses and protecting against pathogens (El-Son et al., 2025; Rimoldi et al., 2023; Wang et al., 2025; Zhang et al., 2024). A balanced intestinal microbiome also contributes to growth efficiency, disease resistance, and stress adaptation (Huang et al., 2025; Liu et al., 2025; Medina-Félix et al., 2023; Tay et al., 2025; Vargas-Albores et al., 2021).

Meanwhile, fish gut microbiota is also influenced by the host and the surrounding environment. (Sadeghi et al., 2023). Studies have shown the influence of host-associated factors, such as host species, age, health status,

and trophic level (an organism's position in a food chain) (Huang et al., 2020; Kim et al., 2021; Zhao et al., 2020). Environmental factors, including diet, rearing systems or habitat, water temperature, and water salinity, have also been reported as major drivers of gut microbial changes (Huyben et al., 2018; Kim et al., 2021; Singh et al., 2025; Zhao et al., 2020). Apart from these methodological factors, such as the DNA extraction method and sampling method, the fish gut microbiota is also significantly influenced (Larsen et al., 2015; Thormar et al., 2024), which poses obstacles when comparing the results of different studies with diverse experimental setups. Thus, it is important to disentangle the impacts of different host factors, environmental factors and various methodological factors. Consequently, understanding and managing gut microbial communities is essential in the development of alternative and functional feeds, as diet-induced changes in microbiota composition can directly impact fish growth, health, and overall aquaculture productivity. It is also important to understand the methodological aspects and their influence when interpreting the microbiota data.

1.4.3 Analytical methods

16S rRNA gene amplicon sequencing is a molecular technique used to profile bacterial communities by targeting and amplifying the 16S ribosomal RNA gene, which contains conserved and hypervariable regions suitable for taxonomic identification (Callahan et al., 2019). The V3-V4 region, one of these hypervariable regions, is commonly amplified in microbial studies because it provides sufficient resolution to differentiate bacterial taxa while remaining compatible with current sequencing technologies (López-Aladid et al., 2023). Illumina and Nanopore are two widely used next-generation sequencing platforms for 16S amplicon analysis: Illumina generates highly accurate short reads suitable for large-scale community profiling, whereas Nanopore produces longer reads, allowing more complete sequence coverage and real-time analysis (Bejaoui et al., 2025; Wang et al., 2021). High-throughput sequencing offers several advantages over traditional Sanger sequencing, including the ability to sequence thousands to millions of reads per run, increased sensitivity for detecting rare taxa, and lower cost per sample, facilitating comprehensive microbial community analyses (Churko et al., 2013). The growing application of these approaches reflects advances in sequencing technology, bioinformatics, and reduced costs, alongside increasing recognition of the ecological and functional

significance of microbiomes, making detailed microbial community characterization more feasible and informative than in the past.

The fish core gut microbiota refers to the group of microbial taxa consistently present across individuals of a species, regardless of external conditions, and is considered essential for maintaining host health and physiological functions (Wu et al., 2024). In rainbow trout, the core gut microbiota is predominantly composed of members of the phyla *Firmicutes* and *Proteobacteria*, with *Mycoplasma*, *Aeromonas*, *Clostridium*, *Deefgea*, *Streptococcus*, *Cetobacterium*, *Lactobacillus*, *Lactococcus*, *Methylobacterium*, *Corynebacterium*, *Shewanella*, and *Staphylococcus* identified as core genera (Hines et al., 2023; Takeuchi & Sugahara, 2025). Notably, *Mycoplasma* species are sometimes the dominant organisms within the microbiome (Hines et al., 2023). Knowledge of the core microbiota provides a baseline for evaluating how alternative feeds, probiotic additives, or environmental stressors affect gut microbial balance. Moreover, differentially abundant microbial groups associated with the treatments can be identified by linear discriminant analysis (LDA) to explain the differences among the treatments (Segata et al., 2011). Identifying these microbial groups provides insight into how specific microbes respond to changes and contribute to host physiology, including digestion, immunity, and growth.

While the core microbiota represents the stable microbial taxa essential for host functions, diversity reflects the adaptability and resilience of the gut ecosystem to changes in diet and environment. Alpha diversity refers to the diversity within a single sample, encompassing the richness, evenness, and phylogenetic breadth of microbial communities (Cassol et al., 2025). Common indices, such as Shannon, Simpson, and Chao1, provide insights into microbial complexity and ecological balance, while Faith's Phylogenetic Diversity (PD) introduces evolutionary relatedness to diversity (Cassol et al., 2025). In contrast, beta diversity measures differences in microbial composition between samples or groups, using metrics such as Bray-Curtis dissimilarity or UniFrac distances to assess community variation (Kers & Saccenti, 2022). Though alterations in alpha or beta diversity do not always indicate a healthy or unhealthy microbiome, alpha and beta diversity analyses together provide complementary insights into gut microbiota (Williams et al., 2024).

Meta-analysis has been defined as "the statistical analysis of a large collection of analysis results from individual studies for the purpose of

integrating the findings" (Glass, 1976). It aims to derive overall estimates or identify consistent patterns, thereby enhancing statistical power and generalizability (Paul & Barari, 2022). Meta-analysis addresses heterogeneity across studies, synthesizes evidence, estimates effect sizes and moderators, and resolves inconsistencies in the literature (Russo, 2007). Its advantages include increased precision, the ability to explore moderators through subgroup or meta-regression analyses, and the capacity to summarise extensive evidence (Paul & Barari, 2022). Limitations include potential publication bias, variability in study design and data quality, and the risk of propagating errors if flawed studies are included (Berman & Parker, 2002).

1.5 Yeast probiotics

Yeasts are unicellular eukaryotic microorganisms that have gained increasing attention as probiotic candidates in aquaculture, due to their diverse physiological traits and metabolic versatility (Hassan et al., 2025; Mahdy et al., 2022). Unlike bacteria, yeasts are naturally tolerant to harsh conditions such as low pH, bile salts, and high salinity, which allows them to survive passage through the gastrointestinal tract of fish (Diguță et al., 2022). Their cell walls are rich in β-glucans, mannans, and chitin, structural polysaccharides that act as PAMPs, stimulating host immune receptors and enhancing innate defences (Shadrack et al., 2021; Zhang et al., 2020). In addition, yeasts synthesize and release bioactive molecules such as vitamins (B-complex and vitamin D precursors), amino acids, nucleotides, organic acids, and extracellular enzymes that contribute to better nutrient absorption, digestion, and metabolic efficiency in fish (Agboola et al., 2021). They also produce antioxidants and pigments (e.g., carotenoids and glutathione) that can contribute to protect fish against oxidative stress and support skin and flesh pigmentation, traits valued in aquaculture (Diguță et al., 2022; Shadrack et al., 2021). Moreover, yeasts can exhibit antimicrobial activity, either by competing with pathogens for adhesion sites and nutrients or by producing killer toxins, fatty acids, and other inhibitory compounds (Drider et al., 2024). Apart from those, they can also modulate the mucosal immune system on the skin, gills and intestine, contributing to stronger barrier function and enhanced resistance to bacterial and viral infections (Caruffo et al., 2015).

Despite their proven benefits, the use of yeast probiotics in fish presents several challenges. Strain-specific variability in functional properties and colonization ability can lead to inconsistent performance across host species and environmental conditions (Merrifield et al., 2010). The viability of yeast cells may also be reduced during feed processing, storage, or passage through the gastrointestinal tract, thereby limiting their probiotic efficacy (Hai, 2015). In some cases, high inclusion levels can disrupt gut microbial balance or overstimulate immune responses, resulting in physiological stress or reduced nutrient assimilation (Gómez et al., 2008; Nayak, 2010). Moreover, limited understanding of host-microbe interactions, colonization mechanisms, and optimal dosing strategies constrains consistent application in aquaculture systems (Hai, 2015; Merrifield et al., 2010). Therefore, while yeast-based probiotics hold substantial promise as functional feed additives, their efficacy depends on careful strain selection, formulation stability, and species-specific validation under aquaculture conditions (Nayak, 2010).

Debaryomyces hansenii, *S. cerevisiae*, and *Cyberlindnera jadinii* are well-studied and promising probiotics for aquaculture, offering complementary benefits to fish health and performance. *S. cerevisiae* has been reported as beneficial in growth, intestinal morphology, immune responses, and antioxidant defences in fish such as striped catfish (*Pangasianodon hypophthalmus*) and European seabass (*Dicentrarchus labrax*) (Boonanuntanasarn et al., 2019; Dawood et al., 2021). *D. hansenii* has shown strong potential in marine fish, such as gilthead seabream (*Sparus aurata*) and longfin yellowtail (*Seriola rivoliana*), where it improves growth, gut microbiota composition, intestinal integrity, mucosal immunity, and stress tolerance (Hernández-Contreras et al., 2021; Sanahuja et al., 2023). More recently, a high-protein yeast, *C. jadinii*, has been investigated in zebrafish (*Danio rerio*), where dietary inclusion modulated intestinal proteome profiles linked to innate immune regulation, suggesting a role in strengthening host defences (Purushothaman et al., 2024).

Rhodotorula, *Rhodosporidium*, and *Sporidiobolus* are genera of pigmented yeasts with growing potential as probiotics in aquaculture, primarily due to their ability to produce carotenoids (β-carotene, torulene, torularhodin) and other antioxidants that enhance fish pigmentation, oxidative balance, and stress resilience (Hof, 2019; Kot et al., 2021; Doan et al., 2023). *Rhodotorula spp*. are also known for generating extracellular enzymes and antimicrobial metabolites, supporting digestion and inhibiting

pathogenic bacteria in the gut (Becerril-Cortés et al., 2022; Bogusławska-Wąs et al., 2019; Fernández-Pacheco et al., 2021; Kaewda et al., 2025; Li et al., 2022). While not yet investigated as a probiotic, the teleomorphic form of *Rhodotorula spp.*, classified as *Rhodosporidium*, is morphologically indistinguishable from *Rhodotorula* in the yeast phase. Sharing most traits with the *Rhodotorula* members, *Rhodosporidium babjevae* also produces lipids and carotenoids with antioxidant and antimicrobial properties, with potential as probiotics in fish (Kim et al., 2012; Sen et al., 2017). Similarly, *Sporidiobolus roseus* is notable for its high torulene and torularhodin content, pigments with strong antioxidant capacity and possible immunomodulatory roles, yet hardly studied as potential probiotics (Linh et al., 2025).

Y. lipolytica, *Pichia kudriavzevii*, and *K. marxianus* are emerging non-Saccharomyces yeasts with promising applications in aquaculture. *Y. lipolytica* is notable for its strong lipid metabolism and ability to produce organic acids, enzymes, and vitamins, supporting lipid digestion, nutrient absorption, and immune stimulation in fish, with potential to enhance growth and resilience (Alamillo et al., 2017; Reyes-Becerril et al., 2021). *P. kudriavzevii* is highly acid- and bile-tolerant, producing organic acids, killer toxins, and antimicrobial compounds, making it a good candidate for pathogen inhibition, gut colonization, and feed efficiency improvement (Agpoon et al., 2024; Lata et al., 2022). Despite the promising traits, it has not been investigated *in vivo* as a live yeast additive in fish. *K. marxianus* is a robust, thermotolerant, fast-growing yeast known for its production of antimicrobial peptides, enzymes, and antioxidants (Bilal et al., 2022; Youn et al., 2022). It has demonstrated immunomodulatory and gut protective effects in poultry (Wang et al., 2017) and mammalian models (Li et al., 2023; Youn et al., 2023), and has also been evaluated in Atlantic salmon and Red-Stirling tilapia (*Oreochromis niloticus var. Stirling*) with no adverse effects on growth or digestibility (Øverland et al., 2013; Ribeiro et al., 2014). Despite the promising attributes, the research mainly focuses on their effects as single-cell proteins, with scarce information regarding their potential as probiotic supplements in fish.

1.6 Fish growth and nutrient digestibility

Growth of fish is a key measurement of net nutrient deposition and overall performance when evaluating a new dietary ingredient. Growth reflects the end result of many physiological processes that include nutrient intake, digestion, absorption, and utilization after meeting requirements for maintenance metabolism (Hardy & Kaushik, 2021). One of the most important aspects of growth performance is its ability to reflect changes in dietary nutrient content (He et al., 2024). Conventionally, growth is assessed by measuring the length and weight of the fish at the beginning and at the end of the feeding period. In addition, more interval measurements at one or several timepoints may be used to learn about the growth condition of the fish over time and adjust feeding rates. Based on the initial weight and the final weight of fish, weight gain (WG) can be expressed in grams or percentages for different conditions. Given the length of the feeding period, specific growth rate (SGR) can also be calculated to express growth as a percentage increase per day, accounting for initial body size. The viscero-somatic Index (VSI) is the ratio of the fish's total viscera weight to its total body weight, reflecting the health of the digestive organs, while the hepato-somatic Index (HSI) is the ratio of the liver's weight to the total body weight, indicating the liver's size and energy reserve status. Apart from growth, the feed conversion ratio (FCR) can be calculated to indicate feed efficiency, with the total weight of the feed consumed during the experimental period.

Digestibility of a nutrient or energy is a measure of the amount of the ingested nutrient absorbed by an animal compared to how much is excreted in the faeces. Nowadays, diets are mainly formulated based on digestible nutrients and energy rather than crude or gross values (Cho & Kaushik, 1990). Digestibility is of great importance in evaluating new feed ingredients for aquaculture (Glencross et al., 2007), and can be determined either by direct or indirect methods. The direct method relies on the total collection of faeces from an animal, which is relatively easy for terrestrial animals, yet more challenging for fish since they are in an aquatic environment. As a result, an indirect method is often used for digestibility estimation in fish. With the help of an inert and indigestible marker, such as titanium oxide or yttrium oxide, digestibility can be calculated based on the concentrations of the marker in the feed and faeces. This digestibility information is critical for determining the nutritional quality of new feed ingredients and estimating

nutritional composition in feed formulations that meet the requirements of fish.

1.7 Model systems

Though *in vivo* feeding experiments provide realistic data, they are resource-intensive, time-consuming, and raise ethical concerns. Artificial gut systems and brine shrimp (*Artemia franciscana*) models offer several methodological and ethical advantages over conventional fish feeding trials. These approaches significantly reduce experimental costs, time, and the number of animals required, aligning with the 3Rs (Replacement, Reduction, and Refinement) principles of animal experimentation. Moreover, they facilitate high-throughput screening of feed ingredients, probiotics, or bioactive compounds before validation *in vivo*. Together, artificial gut and *Artemia* systems provide powerful complementary tools that improve experimental efficiency, reproducibility, and mechanistic understanding of feed-host-microbe interactions prior to large-scale fish trials.

1.7.1 Artificial gut system

An *in vitro* artificial gut system is designed to simulate the physiological, microbial, and biochemical conditions of the gastrointestinal tract of humans as well as animals (Drake & Brogden, 2002; Heimes et al., 2024). It enables the study of digestive processes, nutrient absorption, enzyme activity, and interactions between gut microbiota and dietary components under controlled conditions. A chemostat in batch mode is composed of a continuously stirring bioreactor with the daily addition of media to simulate feed consumption and nitrogen air is continuously bubbled in to maintain an anaerobic environment (Ziv et al., 2013). By replicating factors such as pH, temperature, transit time, and enzymatic secretions, artificial gut systems allow researchers to evaluate the effects of feed additives, probiotics, prebiotics, and other functional ingredients on gut health and nutrient utilization without the need for live animal experimentation (Gościniak et al., 2022). These systems provide a valuable platform for screening diets, understanding host-microbe interactions, and optimizing aquaculture feeds while reducing costs and ethical concerns associated with *in vivo* trials.

1.7.2 Brine shrimp model

The brine shrimp has emerged as a valuable invertebrate model organism in aquaculture and microbiome research, particularly for studying host-microbe and host-diet interactions under controlled conditions (Azra et al., 2022). Its gnotobiotic rearing system enables complete microbial control, allowing researchers to assess the specific effects of dietary components, probiotics, or pathogenic bacteria on host physiology and immunity without interference from undefined microbial communities (Huynh, 2017; Marques et al., 2005). *Artemia* possesses a relatively simple digestive and immune system that shares key innate immune mechanisms with vertebrates, including the production of antimicrobial peptides and the activation of pattern recognition receptors (Sui et al., 2023). Its transparent body facilitates non-invasive visualization of infection dynamics and immune responses, while its rapid life cycle, low maintenance cost, and high reproducibility make it suitable for high-throughput screening of functional feed ingredients and microbial strains prior to validation in fish models (Azra et al., 2022). Consequently, the brine shrimp model represents an ethically sound, cost-effective, and mechanistically informative tool for preliminary evaluation of feed additives and microbial interactions in aquaculture nutrition research.

2. Aims of the thesis

This thesis aimed to determine the contribution of host-associated, environmental, and technical factors that influence the gut microbiota of Atlantic salmon (*S. salar*) and rainbow trout (*O. mykiss*) in freshwater, and to evaluate the potential of several fungal species as either alternative protein sources or probiotic supplements for rainbow trout by investigating their impact on fish growth, nutrient digestibility, gut microbiota, and immune responses. Publicly available datasets containing gut microbiota data and metadata were systematically analysed to assess how various factors influence salmonid gut microbiota, and to provide a basis for future studies to predict outcomes. Diets partially replaced with filamentous fungi were evaluated for their effect on rainbow trout growth, nutrient digestibility, and gut microbiota composition. Moreover, diets supplemented with yeast probiotics were evaluated for their impact on growth, gut microbiota, and immune-related gene expression of rainbow trout.

Specific objectives of this thesis were:

To determine the effect size and rank of host-associated, environmental, and technical factors that influence the gut microbiota of Atlantic salmon and rainbow trout, specifically the alpha and beta diversities, using a meta-analysis (Paper I).

- To evaluate the impact of selected filamentous fungi (*A. oryzae*, *N. intermedia*, *R. delemar*, and *R. oryzae*) on the gut microbiota composition of rainbow trout during an *in vivo* digestibility trial, and to investigate potential interactions with nutrient digestibility and fish performance (Paper II).
- To investigate the impact of yeast probiotic supplements, *R. babjevae* and *K. marxianus*, on rainbow trout growth, gut microbiota, and immune-related gene expression in the gut using an *in vivo* trial (Paper III).

The hypotheses examined in this thesis were:

- Host-associated factors (species and initial weight) and environmental factors (diet and temperature) have a major impact on gut microbiota composition, as differences in fish physiology and rearing conditions influence nutrient availability, metabolic activity, and microbial colonisation dynamics within the host's intestine (Paper I). Moreover, the high abundance of *Mycoplasma* reported in certain studies can be attributed to methodological choices, such as the DNA extraction kit and the selected 16S rRNA hypervariable region, as these technical factors can strongly influence microbial detection and relative abundance estimation (Paper I).
- A 30% inclusion of filamentous fungi in diets fed to rainbow trout induces significant alterations in gut microbiota composition whilst maintaining nutrient digestibility at levels comparable to a reference diet, because fungal biomass provides a balanced profile of bioavailable nutrients and functional compounds that support efficient digestion and modulate the intestinal microbial community (Paper II).
- A 0.5% supplementation of yeast probiotics in diets fed to rainbow trout significantly alters the gut microbiota and the expression of immune-related genes whilst maintaining a growth performance comparable to the reference diet, because yeast-derived bioactive compounds modulate host-microbe interactions and enhance immune responses without compromising nutrient utilisation (Paper III).

3. Materials and methods

3.1 Systematic literature review and meta-analysis (Paper I)

For the selection and collection of raw 16S rRNA sequence data for the meta-analysis in Paper I, all peer-reviewed published papers related to 'salmonid gut microbiota' were identified and manually checked to ensure that they were of high quality and suitable for the subsequent meta-analysis. The potential studies of interest were searched by Title-Abstract-Keyword on SCOPUS using 18 keyword combinations (['salmon', 'trout', or 'char'], ['gut' or 'intestine'], and ['microbiome', 'microbiota', or 'microbe']). Additionally, Title-Abstract were searched on the PubMed database using the same keyword combinations. The combined search from these two databases resulted in 229 full-text research articles published between 1 January 2011 and 31 December 2022. Due to the low number of studies focused on Arctic charr (*S. alpinus*), Chinook salmon (*Oncorhynchus tshawytscha*), and brown trout (*Salmo trutta*), only Atlantic salmon (*S. salar*) and rainbow trout (*O. mykiss*) were selected for Paper I. Further, data collection was limited to *in vivo* studies that sampled the intestinal digesta or mucosa (rather than the whole intestinal tissue) from healthy (no obvious sign of disease or infection) non-triploid and non-selected freshwater salmonids (Atlantic salmon before smoltification and freshwater-raised rainbow trout) in the meta-analysis to reduce the complexity and generalise the results for future research. Moreover, only studies using Illumina MiSeq 16S rRNA gene sequencing were selected as this was the most common sequencing platform at the time compared to other platforms such as Oxford Nanopore. Subsequently, 50 studies were checked for data accessibility due to the combined necessity of clearly stated sample metadata and raw 16S rRNA gene sequencing data required to perform the meta-analysis. As a result, 19 studies meeting the selection criteria (see Paper I for details) were identified for further processing and meta-analysis. All raw 16S rRNA gene sequencing data and sample metadata were downloaded from NCBI Sequence Read Archive (SRA; https://www.ncbi.nlm.nih.gov/sra). All factors that potentially affected the gut microbiota were compiled and categorised for all the studies and they were evaluated regardless if they were specifically addressed in the original study. After the final filtering, 19

studies were selected and only the technical, environmental, and host-associated factors clearly stated in at least 10 studies were analysed in Paper I.

3.2 Experimental design

All studies in Papers II and III were performed on rainbow trout fed with diets containing the following test ingredients: filamentous fungi *A. oryzae* (AO), *N. intermedia* (NI), *R. delemar* (RD), and *R. oryzae* (RO) (Paper II), and the yeast species *R. babjevae* (RB) *and K. marxianus* (KM) (Paper III). Throughout the studies, distal gut content, intestinal tissue, faeces, and feed were sampled for analyses of digestibility (Paper II), gut microbiota (Papers II and III), and gene expression (Paper III; Table 1). The studies were conducted in accordance with the laws and regulations on the use of animals for research purposes in Sweden, overseen by the Swedish Board of Agriculture (diary number: 5.8.18-16347/2017 for Paper II and 5.8.18-23275/2022 for Paper III).

Table 1. Summary of the feeding experiments carried out in Papers I and II of this thesis.

	Paper II	**Paper III**
Fish species	Rainbow trout	Rainbow trout
Initial body weight	75.2 ± 0.8 g	31.3 ± 0.5 g
Period	39 days	32 days
Water temperature	11.5 ± 0.1 °C	13.5 ± 1.5 °C
Number of diets	5	3
Replicates	3	3
Total tanks	15	9
Fungal species	*Aspergillus oryzae*, *Neurospora intermedia*, *Rhizopus delemar*, and *Rhizopus oryzae*	*Rhodosporidium babjevae* and *Kluyveromyces marxianus*
Inclusion rate	30%	0.5%
Feed production	Heat-extrusion	Heat-extrusion and coated with live yeasts in fish oil and rapeseed oil
Material sampled	Distal gut content and faeces	Distal gut content and intestine
Analyses	Fish growth, nutrient digestibility, and gut microbiota	Fish growth, microbiota in gut and feed, and gene expression

3.3 Fish and facilities

The experiments in Papers II and III were conducted at the Aquatic Facility of the Centre for Veterinary Medicine and Animal Science at the Swedish University of Agricultural Sciences (SLU; Uppsala, Sweden). For Papers II and III, the fish were acquired from commercial producers Älvdalslax AB (Älvdalen, Sweden) and Vilstena fiskodling AB (Fjärdhundra, Sweden), respectively, and raised in 500-1000 L square holding tanks. Prior to the feeding experiments, the fish were fed a commercial diet (Nutra, Skretting AS, Stavanger, Norway) at a 2% feed ration during the acclimation periods of 14 and 30 days in Papers II and III, respectively. In the experiments, juvenile rainbow trout with mean initial weights of 75.2 ± 0.8 g (SD) in Paper II and 31.3 ± 0.5 g in Paper III were randomly distributed between 200 L experimental tanks, with n = 18 and 25 fish per tank in Papers II and III, respectively. Experimental tanks were equipped with a partial water recirculation system, and the water was supplied to each tank at a rate of 4 L/min. Each of these tanks were connected to a feed waste and faeces collection system (Hølland teknologi, Sandnes, Norway). Water temperature and dissolved oxygen concentration were measured bi-daily using a handheld measuring instrument (HACH, Sköndal, Sweden). During the experiments, the temperature, dissolved oxygen concentration, and light cycle were kept at 11.5 ± 0.1 °C, 9.7 ± 0.1 mg/L, and 12h:12h (light: dark); and 13.5 ± 1.5 ℃, 9.5 ± 1.0 mg/L, and 12h:12h (light: dark) in Papers II and III, respectively.

3.4 Production of the test ingredients

3.4.1 Filamentous fungi

Two edible Ascomycete fungi, AO CBS 819.72 and NI CBS 131.92, and two Zygomycetes, RD CBS 145940 and RO CBS 112.07, were used in Paper II. The fungi were obtained from the University of Gothenburg microbial culture collection (Gothenburg, Sweden), and the cultivation was carried out at the Swedish Centre for Resource Recovery, University of Borås (Borås, Sweden). Potato dextrose agar (PDA) plates composed of 4 g/L potato extract, 20 g/L glucose, and 15 g/L agar were used for spore activation. Once the plates were inoculated with each individual filamentous fungal spore, they were incubated at 30°C for 3-5 days, followed by storage at 4 °C until

they were used as seed inoculum for cultivation. For preparation of the inoculum solution, 20 mL of sterile distilled water was added to each PDA plate, and the spores were subsequently released through gentle agitation and by scraping the culture surface with the spreader. 500 µL of this supernatant mixture was added to each of the 250 mL shake flasks containing 100 mL of cultivation medium, which was incubated for inoculation.

In Paper II, the thin stillage, a liquid fraction of the side stream from the Bioethanol production, was obtained from Lantmännen Agroetanol AB (Norrköping, Sweden) and stored at -18 °C until it was used as a cultivation substrate. Prior to cultivation, it was sterilised using an autoclave at 121 °C for 20 minutes and 15 PSI of pressure to eliminate any microbial contamination. The pH of all samples was adjusted to 5.50 by adding 10M sodium hydroxide (NaOH) solution. The thin stillage was diluted to 75% w/w with distilled water and later used as a fungal cultivation substrate. The dilutions were carried out by adding the distilled water to the thin stillage.

Cultivation was performed in a demo-scale reactor possessing a 1200 L capacity (4 m high × 0.65 m diameter, Process & Industriteknik AB, Kristianstad, Sweden). First, the reactor was in-situ sterilised at 121 °C for 20 min, using a steam injector. Following this, the diluted thin stillage substrate was transferred to the reactor and subsequently heat-sterilised at 121 °C for 20 min using a steam injector. After cooling the reactor, the pre-grown fungi were transferred to the demo-scale reactor. The fungi were cultivated at 35 °C with an aeration rate of 0.5 vvm for 48 h. Every 12 h, 250 mL of sample was collected to accumulate fungal biomass. The pH during the cultivation was tested and manually adjusted to be between 4.5 and 5.0 using 32% NaOH solution. After the final harvesting, the fungal biomass was compressed until no water came out from the biomass, and it was then dried at 70 °C until the weights were constant. All samples, including biomass and substrate, were stored at -18 °C until analysis.

3.4.2 Yeasts

The toxicity of ten yeast species, *D. hansenii* (DH), *Rhodotorula mucilaginosa* (RM), RB, *Rhodotorula glutinis* (RG), *S. cerevisiae* (SC), *Y. lipolytica* (YL), *C. jadinii* (CJ), KM, *P. kudriavzevii* (PK), and *S. roseus* (SR), were tested in a brine shrimp (*A. franciscana*) model, by evaluating their performance in protecting brine shrimps against *Vibrio parahaemolyticus* infection. The two yeast strains RB CBS 7808 and KM

CBS 6556 were selected based on positive results from this pilot study. The yeast strains were cultivated on yeast-extract peptone dextrose (YPD) medium containing 10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose. The yeast cells were then incubated at 25 ℃ in bioreactors at the Department of Molecular Sciences (SLU, Uppsala, Sweden) for approximately 4 days during each production cycle. At the end of each cycle, the yeast biomass was harvested by centrifugation at 3000 rpm for 10 min. The collected biomass was immediately stored at 4 ℃ (for less than 3 h), after which it was used to coat the feed pellets in Paper III.

3.5 Brine shrimp challenge experiment

Axenic brine shrimp larvae were obtained following the standard decapsulation and hatching process (Baruah et al., 2017; Zheng et al., 2021). In summary, 200 mg of brine shrimp cysts were hydrated in 18 mL of sterile distilled water for 1 h with aeration. Following this, sterile cysts were decapsulated by adding 660 µL NaOH (32%) and 10 mL NaClO (50%). The decapsulation process was brought to a halt after 2 min by using 14 mL of 10 g/L $Na_2S_2O_3$. The aeration was then terminated, and the decapsulated cysts were washed with autoclaved seawater containing 35 g/L of instant ocean synthetic sea salt. All manipulations were conducted under a laminar flow hood. Afterwards, the cysts were suspended in triplicate 50 mL tubes containing 35 mL of autoclaved seawater and incubated for 40 h on a rotor at 6 rpm at 28°C with constant illumination of approximately 2000 lux.

After hatching, the larvae at instar II stage (when they started ingesting particles) were randomly collected (n = 25) and transferred to fresh, sterile 40 mL glass tubes containing 25 mL of autoclaved seawater. The glass tubes with axenic larvae were added with yeast cells of increasing concentrations (0, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7 cells/mL) with 5 replicates per concentration and fed once with 10^7 cells/ml autoclaved LVS3 (*Aeromonas hydrophila*). After coincubation on a rotor for at least 1h, the larvae were challenged with *V. parahaemolyticus* bacteria at 10^7 cells/mL. One group was not challenged to serve as a control. The number of surviving brine shrimps was recorded after 48 h from the start of the challenge.

3.6 Diets and feeding

Feed was prepared at SLU's Feed Technology Laboratory (Centre for Veterinary Medicine and Animal Science, SLU, Uppsala, Sweden) in both experiments. In Paper II, a commercial-like reference diet (Ref diet) was formulated to meet or exceed the nutritional requirements of rainbow trout (National Research Council, 2011). The four experimental diets were formulated as a mixture of the control diet and a test ingredient (AO, NI, RD, or RO) in a 70:30 ratio (Cho & Slinger, 1979). All diets were supplemented with an inert marker, titanium dioxide (TiO2), to determine nutrient digestibility (details regarding the dietary recipes and amino acid profiles are available in Paper II). The dietary preparation started with mixing the dry ingredients for each diet with a paddle mixer (Elektro Helios AB, Stockholm, Sweden) for 30 min. The diets were extruded using a twin-screw laboratory extruder KETSE 20/40 (Brabender GmbH & Co. KG, Duisburg, Germany) with a direct water injection and a Coperion-K-Tron loss-in-weight feeder K-ML-D5-KT20 (Coperion GmbH, Stuttgart, Germany). Water was supplied to the extruder barrel through a peristaltic liquid dosing pump AgnThos 120U (AgnTjo's AB, Lidingö, Sweden). The temperature profiles of the five extruder barrel sections and associated production parameters used during the extrusion are presented in Paper II. Each diet was dried for 2 h at 60 °C in an air-assisted drying oven (Elvärmedetaljer, Skurup, Sweden), and the oil was added using a GVC-10-mini vacuum coating system (Amandus Kahl GmbH & Co. KG, Reinbek, Germany).

As with Paper II, a control diet was formulated with characteristics similar to a commercial diet in Paper III. The feed was produced and coated with freshly harvested yeast cells in two batches to keep the yeast probiotics in the diets alive and fresh (see Paper III for details). In each feed production, the feed was produced by heat extrusion, divided into 3 portions, and then coated separately. The control diet (CTL diet) was only coated with oils (no yeast probiotics) using a vacuum coater (Amandus Kahl GmbH & Co. KG, Reinbek, Germany). For each experimental diet, one portion of the feed was coated with an inclusion of either *R. babjevae* (RB diet) or *K. marxianus* (KM diet). Briefly, the harvested yeast biomass was thoroughly mixed with an equal weight of phosphate-buffered saline (PBS) and then emulsified with the help of an emulsifier, namely soy lectin (0.5g/kg feed), in an oil mixture which was gently shaken in a capped glass bottle. Subsequently, the feed was coated with the mixture in the GVC-10-mini vacuum coating system

(Amandus Kahl GmbH & Co. KG, Reinbek, Germany). All three diets were stored at 4 ℃ both before and during the feeding trial. Data on feed composition, proximate analysis, and amino acid profile is presented in Paper III.

The fish were fed through automatic belt feeders (Hølland teknologi, Sandnes, Norway) twice daily with the assigned experimental diets throughout the 39-day feeding trial in Paper II and the 32-day feeding trial in Paper III. In both experiments, feed was provided at a 2% ratio of the total biomass for each tank on day 1 and the rations were later corrected based on collected feed waste to allow feeding satiation during the entire trial.

3.7 Sampling of feed, faeces, and tissues

Following the preparation of the experimental diets and test ingredients in Papers II and III, samples of each feed were collected in Paper II (Ref, AO, NI, RD, and RO diets) and the control feed (CTL diet) in Paper III. In Paper II, faeces were collected twice per day from each tank using automated belt collectors (Hølland teknologi, Sandnes, Norway). After uneaten feed pellets were separated, faeces and feed waste were stored at -20 °C until analysis. In Paper III, feed waste was separated from the faeces and stored in the corresponding containers at -20 ℃ approximately 2 h after each feeding. After the experiment, the feed, feed waste, and faeces samples were freeze-dried, ground into a powder, and stored at -20 °C for subsequent analysis. Additionally, three feed samples were taken from each batch of every probiotic feed (RB and KM diets) at three respective sampling points, and the yeast viability was monitored throughout the experiment (Details are provided in Paper III).

Initial and final body weight and length of the fish from each treatment were measured at the start and end of the feeding trial to determine growth parameters. In Paper II, the fish intestinal samples were only collected on the last day, whereas in Paper III, the fish intestinal samples were obtained halfway (middle sampling) and at the end (final sampling) of the trial. Before each handling, fish were anesthetised with 60 mg/L tricaine methanesulfonate (MS-222, Western Chemical Inc., Ferndale, WA, United States).

For gut microbiota analyses in Papers II and III, the fish skin was disinfected with 75% ethanol before the dissection and exposure of the

intestine and the entire sampling process was conducted aseptically using a sterile dissection kit. The distal intestine was carefully dissected from 0.5 cm after the ileo-rectal valve to 0.5 cm above the anus, and the distal intestinal content was collected in a sterile tube. In Paper III, the intestinal tissue was also collected in a separate sterile tube for immune function analysis. The samples were snap-frozen in liquid nitrogen and stored at -80 ℃ before DNA and RNA extractions.

In the final sampling in Paper III, another three fish from each tank were also dissected in the same manner, and the digesta of the distal intestine were collected, serially diluted, and plated on YPD agar plates (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, and 20 g/L agar) in duplicate to evaluate the gut colonisation of yeasts. The colonies were counted after incubation at 25℃ for 2 days.

3.8 Proximate composition analysis

All proximate composition analyses of Papers II and III, except the amino acid profiling, were conducted at SLU's Department of Applied Animal Sciences (Uppsala, Sweden). To measure the dry matter content in both feed ingredients, diets, and any feed waste, samples were dehydrated in a hot-air oven for 16 hours at 103 °C, and then allowed to cool in a desiccator before being weighed according to standard methods (AOAC, 1995). For the test ingredients, faeces, and diets, crude protein content (nitrogen, N × 6.25) was determined using the Kjeldahl method (Nordic Committee on Food Analysis, 1976), employing a 2020 Digester with a copper catalyst, and a 2400 Kjeltec Analyzer (FOSS Analytical A/S, Hillerød, Denmark). Crude lipid content analysis was determined using a Soxhlet method (1047 Hydrolysing Unit, Soxtec System HT 1043, FOSS Analytical A/S) (Official Journal of the European Union, 2009). Gross energy content was measured through an isoperibol bomb calorimeter (Parr 6300, Parr Instrument Company, Moline, IL, USA). Ash content was evaluated using a muffle furnace at 550 °C for 5 h according to standard methods (AOAC, 1995). Neutral detergent fibre was analysed by the amylase neutral detergent method (Chai & Udén, 1998). Amino acids were analysed by high-performance liquid chromatography at a certified laboratory (Eurofins Biopharma Product Testing Sweden AB, Uppsala, Sweden).

3.9 Analyses

3.9.1 Growth parameters (Papers II and III)

Four growth parameters namely survival rate, percentage weight gain (WG), specific growth rate (SGR), and feed conversion ration (FCR) and two body indices, hepato-somic index (HSI) and viscero-somatic index (VSI) were calculated using the following equations (as-is basis):

Survival rate (%) = [Number of survived fish/Total number of fish] x 100

WG (%) = [Final weight (g) - Initial weight (g)]/ Initial weight (g) x 100

FCR = Net feed intake (g)/Weight gain (g)

SGR = [ln(Final weight) - ln(Initial weight)]/period (days) x 100

HSI = Liver weight (g)/Fish weight (g) x 100

VSI = Viscera weight (g)/Fish weight (g) x 100

3.9.2 Nutrient digestibility (Paper II)

Dietary apparent digestibility coefficient (ADC) was calculated as: $ADC_{diet} = 1 - (F/D \times F_i/D_i)$, where F is percentage nutrient content (or kJ g^{-1} gross energy) in faeces, D is percentage nutrient content (or kJ g^{-1} gross energy) in the diet, and D_i and F_i are percentage digestion indicators for diet and faeces, respectively.

Apparent digestibility coefficient for the test ingredients was calculated as: $ADC_{ingredient} = ADC_{test} + [(ADC_{test} - ADC_{control}) \times (0.7 \times D_{control}/0.3 \times D_{ingredient})]$, where $D_{control}$ is percentage nutrient content (or kJ g^{-1} gross energy) in the control diet (as-is) and $D_{ingredient}$ is percentage nutrient content (or kJ g^{-1} gross energy) in the fungal biomass (as-is basis).

3.9.3 Extraction of DNA and 16S sequencing (Papers II and III)

Digesta samples ($\leqslant$ 200 mg) were homogenised at room temperature in 1 mL InhibitEX buffer and 0.5 g of 0.1 mm silica beads using a bead beater (Precellys Evolution, Bertin Technologies) for 2 cycles of 1 min at 6000 rpm with a 5 min rest period in between. The DNA was subsequently extracted using QIAamp Fast DNA Stool Mini kit (Qiagen Gmbh, Hilden, Germany) according to the manufacturer's instructions. Polymerase chain reactions (PCR) were performed using Phusion High-Fidelity PCR Master Mix (New England Biolabs) to amplify the V3-V4 region from the 16S rDNA using the primers (341F: CCTAYGGGRBGCASCAG and 806R:

GGACTACNNGGG-TATCTAAT). The extracted DNA was sequenced using Illumina NovaSeq6000 at Novogene (Beijing, China).

3.9.4 Bioinformatics analysis (Papers I-III)

In Papers I and II, raw 16S rRNA gene sequences were analysed using the next-generation microbiome bioinformatics platform QIIME2 version 2022.2 (Bolyen et al., 2019) following the guidelines suggested by the developing team. Raw sequences were imported into QIIME2, demultiplexed, end-joined, and denoised using the QIIME2 built-in DADA2 method. In Paper I, to include most samples without compromising the quality of the data, the sequences were trimmed to maintain a minimum quality score of Q25. Samples with <2000 reads and taxa with fewer than 10 reads in that individual study were discarded to focus on higher abundant taxa in Paper I. Following this, the samples without enough replicates ($n < 5$) or proper control groups in Paper I were also excluded. In Paper II, the sequences were trimmed and filtered to maintain a quality score of Q30 and a minimum read of 2000 per sample. The filtered sequences were then taxonomically classified using the classifier trained on the 16S rRNA V3-V4 gene region from SILVA database v138 (Quast et al., 2013) with a 99% confidence level. The classifier was trained using the same primer sequences mentioned earlier following the protocol provided on QIIME2 forum (https://forum.qiime2.org/). Classified sequences were filtered taxonomically to remove mitochondria, chloroplast, *Archaea*, and Eukaryotes. A phylogenetic tree was generated by QIIME2 built-in fast tree command for each study, and the qiime files were converted to biom format for further analysis in R (version 4.2.1) (R Core Team (R Foundation for Statistical Computing), 2022).

The data of Paper III were processed following the standard protocol of Novogene. The data were subsequently filtered to remove the amplicon sequence variants (ASVs) that included unclassified (phylum level), mitochondria, chloroplast, *Archaea*, and Eukaryotes. A phyloseq object was built by R phyloseq package in each study (McMurdie & Holmes, 2013). To reduce the influence of sequencing depth, all samples were rarefied to the lowest sequencing depth whilst maintaining over 2000 sequences per sample, which was 2838 and 28263 sequences per sample in Papers I and II, respectively. In Paper III, two phyloseq (McMurdie & Holmes, 2013) objects were built and separately analysed one for the digesta samples and the other

for the feed samples. They were rarefied to the lowest sequencing depths, which was 5417 and 11805 sequences per sample for the digesta and feed samples, respectively.

A mini meta-analysis was performed to find associations between the results of Paper II and the findings in Paper I, as well as the previous experiments which had a similar experimental design. Of the 19 studies included in Paper I, 3 studies that investigated Swedish rainbow trout (Huyben et al., 2017, 2018, 2019) were selected to be merged with the gut microbiota data from Paper II. The influencing factors were reduced to 5, including paper, diet, target hypervariable region, rearing system, and DNA extraction kit. The samples in the new phyloseq object were rarefied to 2828 sequences per sample and analysed as described above.

The abundances of the genera were visualised by a stacked bar plot by ggplot2 and ggpubr (Alboukadel, 2020; Hadley, 2016) where the low-abundant genera (see the individual papers for details) were classified as "Other". Shannon diversity and other alpha diversities were calculated by the richness estimation function in the phyloseq package, whilst phylogenetic diversity was generated by the picante package (Kembel et al., 2010) using the rooted tree built in QIIME2 after the multichotomies were transformed to dichotomies by R ape (Paradis & Schliep, 2019). The Bray-Curtis distance was calculated and visualised by non-metric multidimensional scaling (NMDS) after 100 permutations. The weighted UniFrac distance matrix was generated by the distance function in phyloseq and then plotted by Principal coordinates analysis (PCoA). Linear discriminant analysis effect size (LDA, LEfSe) (Segata et al., 2011) was performed by microbiomeMarker on genus level (Yang, 2022), with a minimum LDA score (log10) of 2, followed by Benjamini-Hochberg correction (Benjamini & Hochberg, 1995).

3.9.5 Extraction of RNA and qPCR (Paper III)

Intestinal tissue samples (about 30 mg each) were disrupted and homogenised by a handheld homogeniser in 600 µl of Buffer RLT (Qiagen, Germany). The RNA extraction and purification were performed using the RNeasy Mini RNA Extraction Kit (Qiagen, Germany) according to the manufacturer´s protocol. The concentration and purity of the resulting RNA were then measured using NanoDrop ND-1000 (NanoDrop Technologies, Mounchanin, USA). Subsequently, cDNA was synthesised using the RevertAid H Minus First Strand cDNA Synthesis Kit (ThermoFisher,

Lithuania) following the manufacturer's instructions. The cDNA samples were stored at -80 ℃ until use.

All specific primers for qPCR of the reference and targeted genes were synthesised by Eurofins Biopharma Product Testing Sweden AB (Uppsala, Sweden). In all experimental samples, qPCR amplification of two reference genes [elongation factor 1 (*ef1*), beta actin (*β-actin*)], and six target genes [interleukin-1 beta (*il-1β*), tumour necrosis factor-alpha (*tnf-α*), toll-like receptor 2 (*tlr2*), transforming growth factor-beta (*tgf-β*), mucin-2 (*muc2*), and occludin (*ocln*)] were performed using a StepOnePlus Real-Time PCR machine (ThermoFisher, Massachusetts, USA). Each 25 µL reaction included Maxima SYBR Green/ROX qPCR Master Mix (2X) (ThermoFisher, Lithuania) following the manufacturer's instructions. A mock sample comprised of an equal amount of all the samples was used to generate standard curves. The expression of each target gene was normalised using two reference genes and calibrated based on the control samples. The Pfaffl method was used to calculate the relative expression for each gene (Pfaffl, 2001).

3.10 Statistical analyses

The normality of the distribution of residual errors for the microbial alpha diversity indices, nutrient digestibility, growth, and gene expression data were tested using Shapiro-Wilk normality tests (Shapiro & Wilk, 1965). The impact of the factors and the diets on alpha diversity indices was then analysed according to the normal distribution (see individual papers for details). For data following the normal distribution, analysis of variance (ANOVA) was performed, followed by post-hoc Tukey HSD test (Keselman & Rogan, 1977) for significance. For the non-normal distributed data, Kruskal-Wallis tests (Kruskal & Wallis, 1952) were performed, followed by post-hoc Dunn's test (Dunn, 1964). In Papers I and II, alpha diversity values were analysed using generalised linear mixed-effects models by R lme4 (Bates et al., 2015) and car (Fox & Weisberg, 2019) packages to analyse the contribution of the influencing factors and the treatments on alpha diversities with the influence of random effects. In Paper III, the gene expression data were analysed by two-way ANOVA followed by Student's t-test (Student, 1908) corrected by the Benjamini-Hochberg method (Benjamini & Hochberg, 1995).

For beta diversity matrices, permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2017) (9999 permutations were performed) with a weighted Unifrac distance were used to evaluate significance of the influencing factors on beta diversity in Paper I. Pairwise PERMANOVA was performed on all the factors with more than two subgroups to understand the differences between each of the two subgroups. In Paper I, multivariate homogeneity of groups dispersions was also tested by PERMDISP (Anderson, 2006) in which both ANOVA (distances to centroids were calculated) and permutational analysis (999 permutations) were performed. In Papers II and III, both PERMANOVA (Anderson, 2017) and analysis of similarities (ANOSIM) (Clarke, 1993) were applied using 9999 permutations to test the significance of the diets. The p-values <0.05 were considered significant and plots included means ± standard error.

4. Results

4.1 Effects of host-associated, environmental, and technical factors on the gut microbiota (Paper I)

According to the Kruskal-Wallis tests and PERMANOVA, all 15 factors related to the host, environment, and technical methods had significant effects on both alpha and beta diversity of microbes in the gut of Atlantic salmon and rainbow trout (Table 2, Figures 4 and 5).

4.1.1 Effects of host-associated, environmental, and technical factors on the gut microbiota (Paper I)

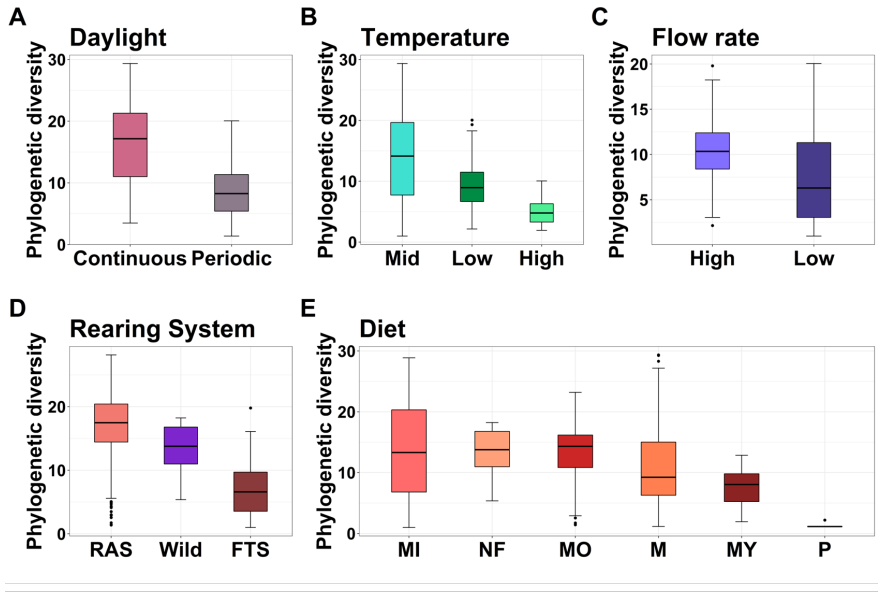
Technical factors heavily influenced the beta diversity (Table 2) and clustering of gut bacteria, whereas their impact on alpha diversity was less strong (Paper I). Paper/study was the most dominant factor regarding both Phylogenetic diversity and weighted UniFrac distance, explaining over 60% of the variance of the beta diversity (Table 2; see Paper I for details). Other technical factors, such as target hypervariable region and DNA extraction kit, explained 24.4% and 19.1% of the variance of beta diversity, which was higher than both the environmental and host-associated factors (Table 2).

4.1.2 Environmental influences on the gut microbiota

In contrast, the environmental and host-associated factors did not explain as much of the beta diversity variance compared to the technical factors. However, between them, the environmental factors explained more variance than the host-associated factors (Table 2). The most explanatory environmental factor was diet which accounted for over 18.6% of the total beta diversity variance, whilst the rearing system, water flow rate, daylight, and the intestinal region explained around 11.6%-15.2% of the beta diversity variance (Table 2). The recirculating system showed significantly higher phylogenetic diversity compared to its wild and flow-through counterparts, whilst the flow-through system had the lowest alpha diversity (Figure 4D; $p < 0.001$, chi-squared = 357.5). The samples collected from the mid-temperature ($p < 0.001$, chi-squared = 126.5; Figure 4B) or high-water flow rates ($p < 0.001$, chi-squared = 220.9; Figure 4C) had significantly higher Phylogenetic diversity values than the others.

Table 2. Impact of influencing factors on the beta diversity of gut microbiota in freshwater salmonid fishes using weighted UniFrac and PERMANOVA. 9999 permutations were performed in each of the PERMANOVA tests to obtain the p value, R square, and pseudo-F values. SGR: specific growth rate, FCR: feed conversion ratio. All p-values < 0.001.

Factor	Factor type	Sample size	R squared	Variance explained (%)	Pseudo-F
Paper/Study	Mixed	783	0.618	61.8	68.57
Target hypervariable region	Technical	783	0.244	24.4	125.90
DNA extraction kit	Technical	783	0.191	19.1	46.00
Diet	Environmental	745	0.187	18.7	29.75
DNA polymerase	Technical	713	0.173	17.3	32.46
Initial weight	Host-associated	706	0.160	16.0	37.02
Rearing system	Environmental	572	0.152	15.2	46.41
Flow rate	Environmental	406	0.141	14.1	64.26
Daylight	Environmental	549	0.123	12.3	54.48
Intestinal region	Host-associated	783	0.116	11.6	51.26
SGR	Host-associated	356	0.110	11.0	48.10
FCR	Host-associated	315	0.090	9.0	38.16
Species	Host-associated	783	0.081	8.1	68.45
Temperature	Environmental	680	0.075	7.5	20.98
Weight gain	Host-associated	193	0.061	6.1	25.51



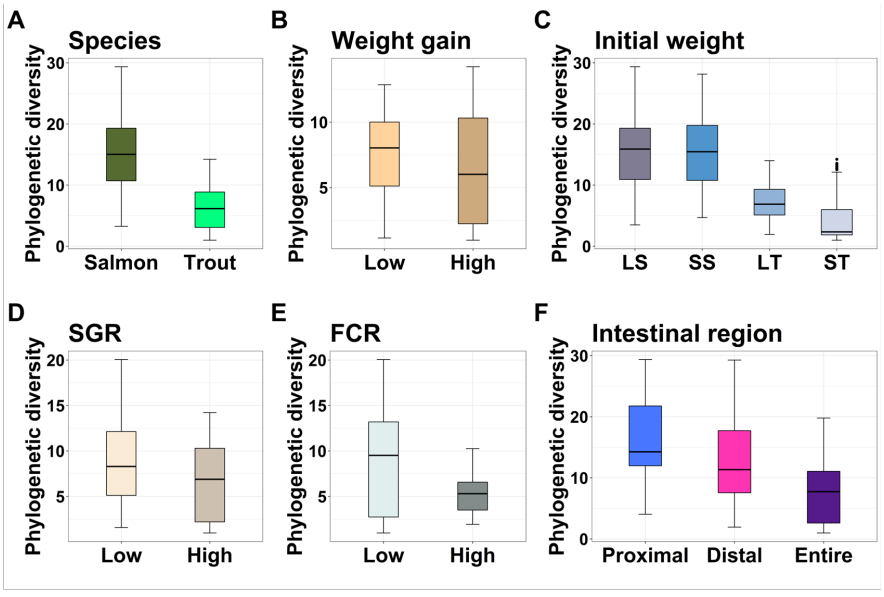
Tag	Factor	p value	Chi squared	Tag	Factor	p value	Chi squared
A	Daylight	< 0.0001	205.5	D	Rearing system	< 0.0001	357.5
B	Temperature	< 0.0001	126.5	E	Diet	< 0.0001	132.6
C	Flow rate	< 0.0001	220.9				

Figure 4. (A) Boxplots of Faith phylogenetic diversity of 783 gut microbiota samples from 19 freshwater salmonid studies (environmental factors). (B) Temperatures lower than 13 ℃ are considered as low temperature, whilst 'High' indicates temperatures higher than 15 ℃. All other temperatures are considered as mid temperatures. (C) Water flow rates higher than 8 L/min were categorised as high, otherwise considered as low. (D) 'RAS' stands for recirculating aquacultural system, whilst 'wild' indicate a wild-like environment, and 'FTS' is the flow-through system. (E) 'M' stands for marine-based feeds. 'MI', 'MY', and 'MO' indicate marine-based feeds with inclusions of insects, yeasts, and other nutrient sources such as other prebiotics or oils. 'P' indicates plant-based feeds, whereas 'NF' indicates a wild-like environment without any feed provided. The table below the plots provides the p-values and chi-squared values from Kruskal-Wallis tests.

4.1.3 Environmental influences on the gut microbiota

Host-associated factors, such as initial weight and species, had only minor influences on beta diversity (Table 2). The most explanatory host-associated factor was initial weight, which accounted for just 16.0% of the variation, whereas the other host factors (i.e., SGR, FCR, species, and weight gain) explained <11.0% of the beta diversity variation (Table 2). Intestinal microbiota in Atlantic salmon had a significantly higher phylogenetic

diversity than rainbow trout (Figure 5A; $p < 0.001$, chi-squared = 375.1). Furthermore, initial weight did not significantly differentiate the Phylogenetic diversity of either the Atlantic salmon subgroup or rainbow trout subgroups despite there being a significant effect and separation by fish species (Figure 5C; $p < 0.001$, chi-squared = 369.4).



Tag	Factor	p value	Chi squared	Tag	Factor	p value	Chi squared
A	Species	< 0.0001	375.1	D	SGR	< 0.0001	192.7
B	Weight gain	< 0.0001	135.4	E	FCR	< 0.0001	209.0
C	Initial weight	< 0.0001	369.4	F	Intestinal region	< 0.0001	114.4

Figure 5. (A) Boxplots of Faith phylogenetic (alpha) diversity of 783 gut microbiota samples from 19 freshwater salmonid studies (host-associated factors). (B) Weight gains exceeding 140 g are regarded as high, otherwise considered as low. (C) 'LS' stands for large salmon weighing at least 40 g whilst small salmon weighing less than 40 g are labelled as 'SS'. 'LT' stands for large trout weighing more than 80 g whilst small trout weighing less than 80 g are labelled as 'SS'. (D) Specific growth rates higher than 1.2 are considered as high, otherwise regarded as low. (E) Feed conversion ratios higher than 2.0 are considered as high, otherwise considered as low. The table below the plots provides the p-values and chi-squared values from Kruskal-Wallis tests.

4.2 Effects of dietary fungi on growth, nutrient digestibility, and gut microbiota (Paper II)

4.2.1 Dietary nutritional composition and growth performance

The five experimental diets tested in Paper II consisted of 43.9-46.8% crude protein, 18.2-20.6% crude fat, 5.1-6.4% ash, and 92.2-94.1% dry matter. For feed formulation, proximate nutrients, and amino acid profile, refer to Paper II. During the 39-day feeding experiment, the fish that were fed the Ref diet achieved the highest mean WG, HSI, and SGR of 90.14 ± 8.35 %, 2.10 ± 0.14, and 1.73 ± 0.12 %/day among the five dietary groups (Table 3), respectively. The fish fed the other diets with fungal biomass inclusions all had lower WG, HSI, and SGR, although no significance was observed. The Ref diet also had the lowest FCR of all five diets, but the difference was not significant (Table 3).

Table 3. Growth parameters and body indices of the fish fed different diets with inclusions of *A. oryzae* (AO), *N. intermedia* (NI), *R. delemar* (RD), and *R. oryzae* (RO). WG: weight gain; HSI: hepato-somatic index; VSI: viscero-somatic index: SGR: specific growth rate; FCR: feed conversion ratio.

	Ref	**AO**	**NI**	**RD**	**RO**
WG (%)	90.14 ± 8.35	73.00 ± 1.25	65.01 ± 3.13	83.00 ± 10.50	73.99 ± 2.76
HSI	2.10 ± 0.14	1.90 ± 0.06	1.77 ± 0.16	1.95 ± 0.23	1.97 ± 0.19
VSI	13.93 ± 0.55	14.28 ±1.00	14.06 ± 1.09	14.51 ± 2.15	13.10 ± 0.55
Survival (%)	100.00 ± 0.00	100.00 ± 0.00	94.44 ± 5.56	100.00 ± 0.00	98.15 ± 1.85
SGR	1.73 ± 0.12	1.48 ± 0.02	1.35 ± 0.05	1.62 ± 0.16	1.50 ± 0.04
FCR	0.67 ± 0.01	0.79 ± 0.12	0.72 ± 0.04	0.72 ± 0.07	0.72 ± 0.04

4.2.2 Nutrient digestibility

For dry matter, the ADC of the Ref diet (84.5%) was significantly higher compared to all other treatments (73.6-78.7%) (Table 4). Similarly, crude protein digestibility was highest for the Ref diet at 94.8%, with all other treatments being significantly lower (85.9% to 89.8%). Gross energy followed the same trend, with the ADC of the Ref diet at 89.2%, which was significantly higher than the other treatments (77.7% to 81.8%). Overall, the standard errors are relatively small, indicating consistent measurements, and

all nutrient digestibility differences among the diets were statistically significant ($p < 0.05$). For the ingredients' ADCs (Table 5), dry matter, crude protein, and gross energy varied among the treatments. Numerically, the lowest ADC was consistently observed for RO diet, whilst the p-values indicated certain differences approaching significance (e.g., dry matter and gross energy with p-values of 0.06).

Table 4. Apparent digestibility coefficient (ADC, %) in the five experimental diets with inclusions of *A. oryzae* (AO), *N. intermedia* (NI), *R. delemar* (RD), and *R. oryzae* (RO) fed to rainbow trout for 39 days (n=3 per diet). The letters labelled as superscripts indicate the significance ($p < 0.05$).

	Ref	AO	NI	RD	RO	Pooled SEM	p-value
Dry matter	84.5^{a}	77.3^{b}	78.7^{b}	75.5^{b}	73.6^{b}	1.09	<0.05
Crude protein	94.8^{a}	89.8^{b}	87.8^{b}	85.9^{b}	88.0^{b}	0.88	<0.05
Gross energy	89.2^{a}	81.8^{b}	82.4^{b}	79.5^{b}	77.7^{b}	1.13	<0.05

Table 5. Apparent digestibility coefficient (ADC, %) of test ingredients in the five experimental diets with inclusions of *A. oryzae* (AO), *N. intermedia* (NI), *R. delemar* (RD), and *R. oryzae* (RO) fed to rainbow trout for 39 days (n=3 per diet). The letters labelled as superscripts indicate the significance ($p < 0.05$).

	AO	NI	RD	RO	Pooled SEM	p-value
Dry matter	60.2	65.6	54.8	48.7	3.83	0.06
Crude protein	76.1	72.9	64.4	71.3	2.43	0.14
Gross energy	61.0	63.0	51.0	45.0	4.10	0.06

4.2.3 Modulation of the gut microbiota

The microbiota composition followed a pattern that could be linked to diet, but certain individual fish possessed large variations in microbial composition (Figure 6). The gut microbiota of the fish fed the Ref diet was primarily dominated by *Weissella* and *Lactobacillus*. Compared with the Ref diet, fish fed the AO diet differed greatly between individuals and the abundance of *Weissella* and *Lactobacillus* were lower. Similar to the Ref diet, fish fed the NI and RD diets showed a high abundance of *Weissella* and

Lactobacillus in the gut. In contrast, fish fed the RO diet had a completely different gut microbiota which predominantly consisted of *Bacillus*.

The fish fed AO ($F = 5.88$, $p = 0.036$), NI ($F = 5.75$, $p = 0.038$), and RD ($F = 14.57$, $p = 0.003$) diets had a significantly different and lower Shannon diversity compared to the Ref diet (Figure 7A), whereas the RO diet did not have a significant effect on alpha diversity. The NMDS analysis based on Bray-Curtis distances showed that fish fed the AO (R square = 0.13, adjusted $p = 0.42$), NI (R square = 0.15, adjusted $p = 0.09$), and RD (R square = 0.11, adjusted $p = 0.24$) diets all clustered on the left together with the Ref diet, whereas fish fed the RO diet (R square = 0.54, adjusted $p = 0.01$) significantly clustered away from the other diets (Figure 7B).

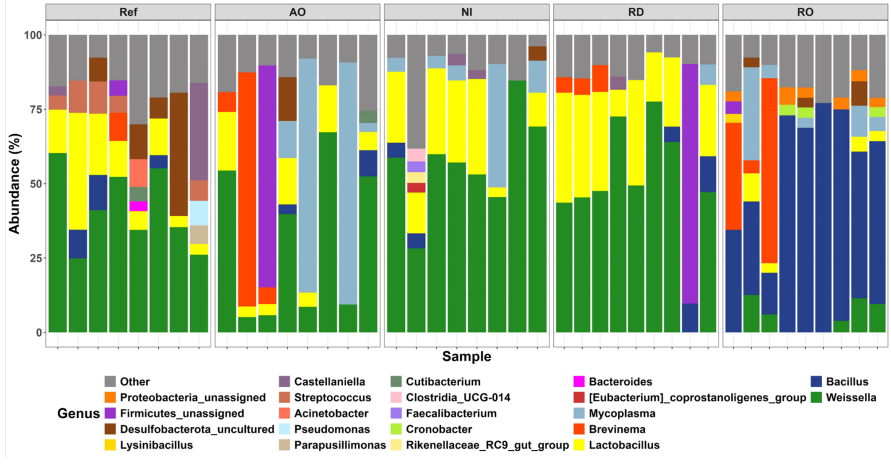


Figure 6. Stacked bar plots illustrating the relative abundance on genus level in the gut of rainbow trout fed a reference (Ref), *A. oryzae* (AO), *N. intermedia* (NI), *R. delemar* (RD), and *R. oryzae* (RO) diet for 39 days (n=9 or 8 per diet). Phyla with a relative abundance lower than 3% per sample are shown as "Other".

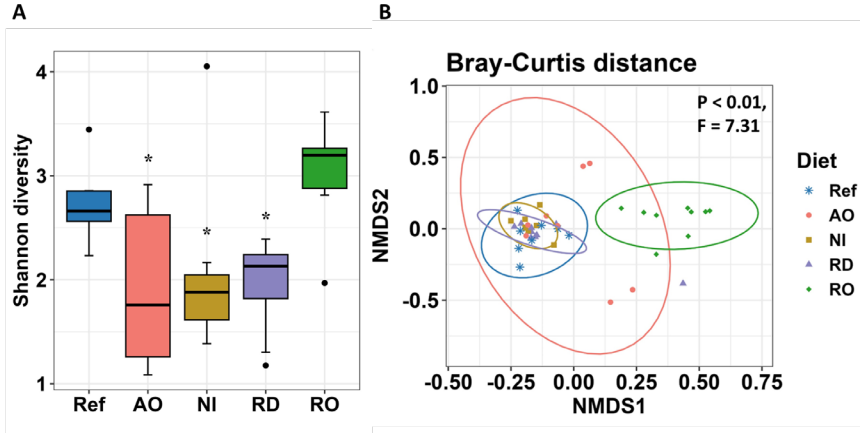


Figure 7. Shannon diversity (A) and Bray-Curtis distance (B) of the gut samples of rainbow trout fed a reference (Ref), *A. oryzae* (AO), *N. intermedia* (NI), *R. delemar* (RD), and *R. oryzae* (RO) diet for 39 days (n=9 or 8 per diet). The star labels (*) above the bars and beside the group names show the significance of the diet compared with the Ref group. The significance level is 0.05 in both plots.

4.3 Effects of yeast probiotic supplements on the growth, gut microbiota, and gene expression (Paper III)

4.3.1 *Artemia* survival

The impact of various yeast species and their application doses was evaluated through a survival study using *A. franciscana* and its pathogen *V. parahaemolyticus* as a model system under axenic conditions. Since yeast is primarily considered an immune stimulant that mainly influences the innate immune system, *Artemia*, as an invertebrate, provided a suitable platform for assessing this effect.

In this experiment, we examined the performance of different yeast species: *D. hansenii* (DH), *R. mucilaginosa* (RM), *R. babjevae* (RB), *R. glutinis* (RG), *S. cerevisiae* (SC), *Y. lipolytica* (YL), *C. jadinii* (CJ), *K. marxianus* (KM), *P. kudriavzevii* (PK), and *S. roseus* (SR) in enhancing the survival of *Artemia* against vibriosis. The survival results showed that priming *Artemia* with KB or RB at a concentration of 10^6 cells/mL led to a significantly higher survival rate ($P < 0.05$) compared to the animals in the

Vibrio group not fed with yeast cells (Figure 8). Similar positive effects were also observed for DH, RM, RG, CI, PK, and SR. However, among all the tested yeasts, RB and KM were selected for subsequent fish experiments due to the reproducibility of results, optimal yeast growth characteristics, and the manageable number of yeast cells required for experimentation.

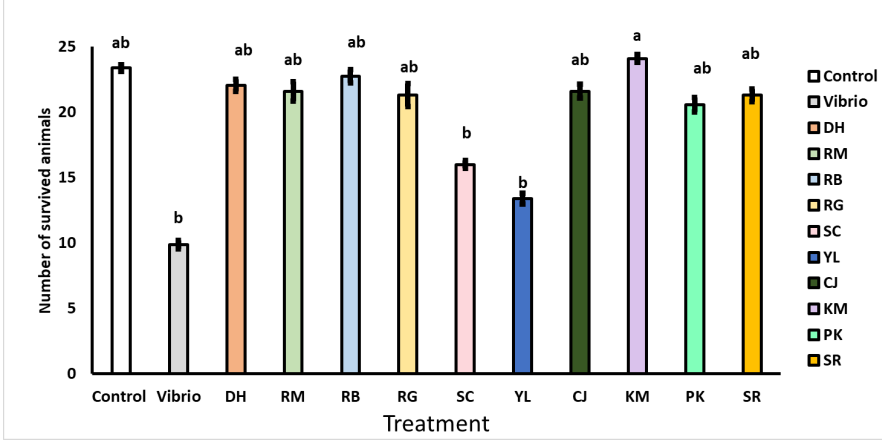


Figure 8. The number of Artemia that survived until the end of the *Vibrio* challenge experiment. The control group was not challenged, whilst the Vibrio group was challenged but not pretreated by yeast. The other groups were challenged and pretreated with yeasts: *D. hansenii* (DH), *R. mucilaginosa* (RM), *R. babjevae* (RB), *R. glutinis* (RG), *S. cerevisiave* (SC), *Y. lipolytica* (YL), *C. jadinii* (CJ), *K. marxianus* (KM), P. kudriavzevii (PK), and *S. roseus* (SR). The letters above the bars indicate the significance ($p<0.05$) by Tukey HSD.

4.3.2 Impact on gut microbiota

Alpha and beta diversity of gut microbiota showed no differences between the probiotic diets and the control diet (see Paper III). Linear discriminant analysis (LDA) was performed to identify differentially abundant microbial groups related to the diets. Significantly higher abundance of *Lactococcus* (Table 6, adjusted $p < 0.001$) was observed in the gut of the fish fed the RB diet, while no microbial group was found to be associated with the fish fed the other diets.

Table 6. Differential abundance of the genera associated with the dietary groups. CTL: control diet, RB: *R. babjevae* diet. Only microbial features with an linear discriminant score (log10) score of 2.0 or higher are shown. The p-values were corrected by the Benjamini-Hochberg method.

Feature	Group	LDA score	p-value	adjusted p-value
Lactococcus	RB	2.16	<0.001	<0.001
Bifidobacterium	RB feed	2.48	<0.001	<0.001
Acinetobacter	RB feed	2.32	0.042	0.042
Blautia	RB feed	2.13	<0.001	<0.001
Subdoligranulum	RB feed	2.03	<0.001	<0.001
Faecalibacterium	CTL feed	2.24	<0.001	<0.001
Streptococcus	CTL feed	2.18	0.008	0.008

4.3.3 Gene expression

Gene expression data showed that both *ocln* and *tgf-β* genes were upregulated in the middle sampling and then downregulated in the final sampling in the gut tissue of the fish fed both the RB and KM diet (Figure 9). Two-way ANOVA confirmed a significant impact of the RB diet on the *ocln* gene expression of the middle sampling fish ($p = 0.016$, $F = 11.0$), with a fold change of 2.1 (Figure 9A). Meanwhile, the *ocln* gene expression of the fish receiving the RB diet in the final sampling was also significantly lower than the middle sampling counterpart ($p = 0.034$, $F = 7.5$), with a fold change of 0.3 (Figure 9A). The expression of *tgf-β* in the gut tissue of the fish of the final sampling was also significant (Figure 9B, $p = 0.027$, $F = 8.5$). Although no significant effect was observed, the expression of *ocln* and *tgf-β* genes in the gut tissue of the fish fed the KM diet was upregulated 1.6 folds in the middle sampling, which decreased to 0.6 and 0.3 fold in the final sampling, respectively (Figure 9B).

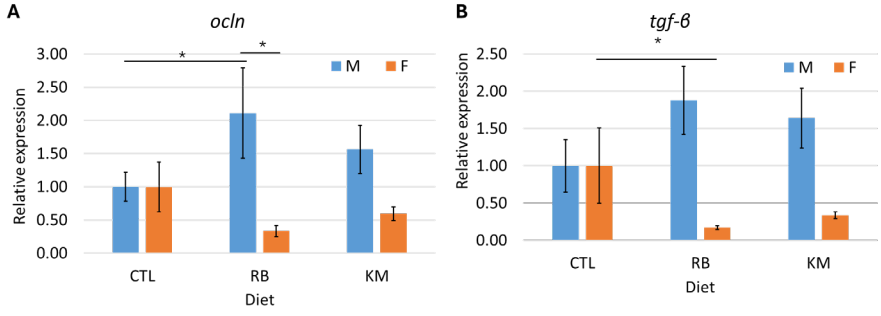


Figure 9. Relative expression of two gut-related genes in the distal intestine tissue of rainbow trout (n=9 per diet) fed the control diet (CTL) and diets supplemented with either *R. babjevae* (RB) or *K. marxianus* (KM). "M" indicates the samples from the middle sampling, whilst "F" indicates the final samples. The stars (*) between the bars indicate significant differences between the groups regarding the relative gene expression levels according to two-way ANOVA, considering both the effects of the diet and the sampling time. Error bars show the standard errors of the means.

4.3.4 Nutritional composition and growth performance

The reference diet tested in Paper III consisted of 49.9% crude protein, 16.3% crude lipid, 8.6% ash, and 95.7% dry matter. Over the 32-day study period, the fish grew with an average weight gain (%) of 41.1 ± 0.5%, 46.0 ± 7.0%, and 39.3 ± 8.6%, which were achieved for the fish fed the CTL, RB, and KM diets (Table 7), respectively, with no significant difference (RB-CTL: $p = 0.252$, $F = 5.709$; KM-CTL: $p = 0.925$, $F = 0.014$) compared to the CTL group. Similarly, no significant difference was detected in feed conversion ratio (FCR, RB-CTL: $p = 0.581$, $F = 0.598$; KM-CTL: $p = 0.625$, $F = 0.446$) and specific growth rate (SGR, RB-CTL: $p = 0.271$, $F = 4.845$; KM-CTL: $p = 0.960$, $F = 0.004$) between the experimental and CTL diets. The FCR and SGR were 1.6 ± 0.2, 1.3 ± 0.2, and 1.8 ± 0.3, and 2.8 ± 0.0% per day, 2.9 ± 0.2% per day, and 2.7 ± 0.4% per day in the CTL, RB, and KM groups, respectively (Table 7).

Table 7. Growth parameters in rainbow trout fed the three diets for 30 days in the 32-day feeding experiment. The tested diets are the control diet (CTL) and diets supplemented with either *R. babjevae* (RB) or *K. marxianus* (KM). Values shown are mean ± standard error of the mean. WG(%) = Weight gain percentage; SGR = Specific growth rate; FCR = Feed conversion ration. Neither the RB nor KM group is significantly different from the CTL group regarding any of the shown parameters.

Parameter	CTL	RB	KM
Initial weight (g)	31.86 ± 0.90	31.42 ± 1.47	30.60 ± 0.64
Final weight (g)	73.01 ± 1.44	77.44 ± 8.40	69.86 ± 9.22
WG (%)	129.25 ± 2.08	145.21 ± 15.82	127.23 ± 25.44
SGR (% per day)	2.77 ± 0.03	2.98 ± 0.22	2.69 ± 0.38
FCR	0.76 ± 0.02	0.72 ± 0.03	0.81 ± 0.11
Survival (%)	97.33 ± 2.67	98.67 ± 1.33	98.67 ± 1.33

4.4 Mini meta-analysis of Papers I-II

A mini meta-analysis was performed to find associations between the results of Paper II and the findings in Paper I, as well as the previous experiments with a similar experimental design. Stacked bar plots (Figure 10) revealed distinct microbial compositions in the fish gut across different papers and diets. The samples from Huyben et al. (2017) and Paper II had a more similar microbiota compared with the other two studies (Figure 10A), with a relatively high abundance of *Weissella* and *Lactobacillus*. After being grouped by diet, the fish fed marine-based diets, marine-based diets with inclusions of filamentous fungi, and marine-based diets with inclusions of yeast showed similar microbial compositions with a relatively high abundance of *Weissella*, *Mycoplasma*, and *Lactobacillus* (Figure 10B). However, the fish fed the insect diets from the Huyben et al. (2019) paper possessed a distinctly different gut microbiota composed of *Pseudomonas*, *Oceanobacillus*, and *Corynebacterium*, which the LDA results also confirmed (Table 8).

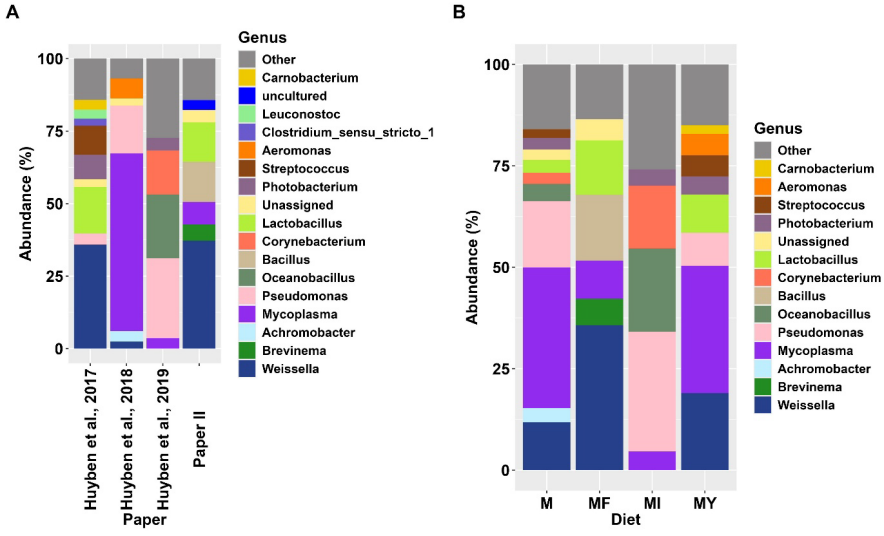


Figure 10. Relative abundance on the genus level of 246 gut microbiota samples from 4 rainbow trout studies in Sweden ((Huyben et al., 2017, 2018, 2019), Paper II). 'M' stands for marine-based feeds. 'MF', 'MI', and 'MY' indicate marine-based feeds with inclusions of filamentous fungi, insects, and yeasts. Only genera with more than 2% abundance are shown in the plots.

Table 8. Differential abundance of the genera associated with the dietary groups. "MF" indicate marine-based feeds with inclusions of filamentous fungi, whilst "MI" indicate marine-based feeds with inclusions of insects. Only microbial features with a linear discriminant score (log10) of 2.0 or higher are presented. The p-values were corrected by the Benjamini-Hochberg method.

Genus	Diet	LDA score	p-value	adjusted p-value
Weissella	MF	2.720	<0.001	<0.001
Bacillus	MF	2.381	<0.001	<0.001
Lactobacillus	MF	2.297	<0.001	<0.001
Pseudomonas	MI	2.690	<0.001	<0.001
Oceanobacillus	MI	2.465	<0.001	<0.001
Corynebacterium	MI	2.350	<0.001	<0.001

All the tested factors had significant impacts on Shannon diversity. Interestingly, Dunn's test revealed significant differences between the Shannon diversity of the samples from Huyben et al. (2017) and those from Huyben et al. (2018) and Huyben et al. (2019) (Figure 11A). However, the

alpha diversity of the samples from Paper II was not significantly different from the samples from Huyben et al. (2017). As for the dietary effect, fish fed marine-based diets with filamentous fungi or yeast inclusions had significantly lower Shannon diversity than the marine-based diets, whilst the diets including insect meal resulted in a slightly higher alpha diversity than the marine-based diets, but with no significant difference (Figure 11B). Meanwhile, the fish fed the fungal diets had a similar Shannon diversity.

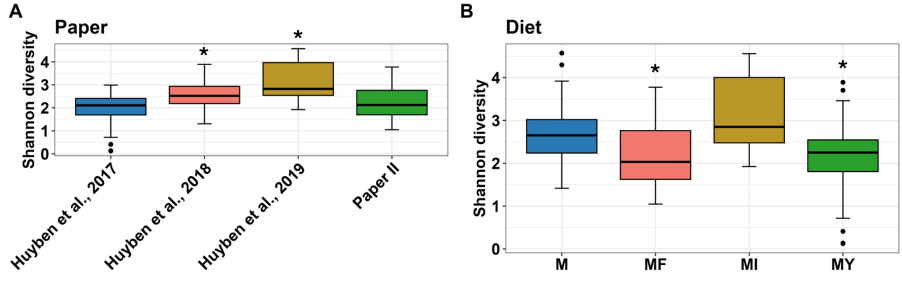


Figure 11. Boxplots of Shannon diversity of 246 gut microbiota samples from 4 rainbow trout studies in Sweden ((Huyben et al., 2017, 2018, 2019), Papers II). In plot B, 'M' stands for marine-based feeds whilst 'MF', 'MI', and 'MY' indicate marine-based feeds with inclusions of filamentous fungi, insects, and yeasts.

Similarly, all the tested factors also had significant impacts on Bray-Curtis distance, and the samples clearly clustered by paper (Figure 12A). The datapoints within each paper are closely related, with relatively small variation. The samples from Huyben et al. (2017) and Paper II clustered in the top right region and overlapped in a common area. In contrast, the datapoints from the other two studies were scattered in the bottom left region and also overlapped in a smaller common area. The differences between the beta diversity of the samples from either two of these four papers were significant. Likewise for diet, the fish fed the marine-based diets and marine-based diets with yeast inclusions were scattered in the centre with large variations (Figure 12B). The fish fed the diets with filamentous fungi inclusions clustered at the top, whilst the fish fed the insect diets clustered at the bottom. The datapoints from the filamentous fungal diet and the insect diet did not overlap with each other, however, they all overlapped with the other two groups in the central area. Similar to paper, the differences between

the beta diversity of the fish fed either two of these four diets were significant.

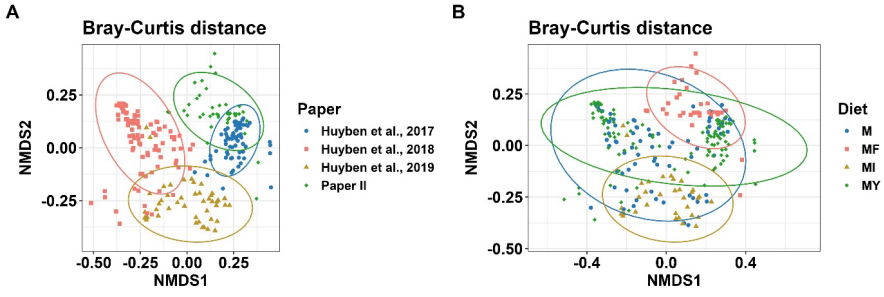


Figure 12. Non-metric multidimensional scaling (NMDS) plots of Bray-Curtis distance of 246 gut microbiota samples from 4 Swedish rainbow trout studies ((Huyben et al., 2017, 2018, 2019), Papers II). Circles represent 95% confidence intervals. In plot B, 'M' stands for marine-based feeds. 'MF', 'MI', and 'MY' indicate marine-based feeds with inclusions of filamentous fungi, insects, and yeasts.

5. Discussion

In this thesis, a series of studies were conducted to evaluate the role of host-associated, environmental, and technical factors on the gut microbiota of Atlantic salmon and rainbow trout using *in silico* analysis of publicly available datasets by applying a rigorous selection criteria. Additionally, the potential of various fungal species as alternative protein sources and yeasts as probiotic supplements for rainbow trout was experimentally evaluated. In this chapter, the key findings and broader implications of these studies are discussed, while detailed results and specific analyses are presented in the individual papers.

5.1 Environmental, host-associated, and technical factors that affect the gut microbiota

5.1.1 Technical influences on the gut microbiota

Paper I indicated that paper/study is the overall most dominant factor in affecting both the alpha and beta diversity of microbiota in freshwater salmonids (Table 2; see details in Paper I). In addition, all the factors that were evaluated in this meta-analysis had a significant effect, which is in line to what was previously reported a meta-analysis on the microbiota of shrimp (Cornejo-Granados et al., 2018). In agreement, a meta-analysis on the gut microbiota of 1,046 healthy humans from around the world found that many factors influenced the beta diversity, similar to the findings in paper I. Specifically the environment (e.g. diet and housing) explained up to 20% of the variation whereas host effects (e.g. ancestry) had minor affects (Rothschild et al., 2018). Aside from the paper/study, other technical factors including target hypervariable region and DNA extraction kit were also dominant factors in shaping the beta diversity of salmonid gut microbiota, but not in shaping the alpha diversity counterpart (Table 2; see details in Paper I). In the reviewed studies testing the effects of target hypervariable regions and DNA extraction kits using the stool samples from human and mice, significant shifts of microbiome composition related to experimental conditions were found (Stinson et al., 2018). In agreement, DNA extraction method have been shown to be an important factor that influence the

identified microbiota composition in studies performed in zebrafish, horse, dog, cat and mice faeces (Hart et al., 2015). By contrast, a study on human faeces found that DNA extraction method had little effect on microbiota communities while the target hypervariable region had an immense impact (Rintala et al., 2017). The results shown in the present study revealed that both DNA extraction method and target hypervariable region had a high impact on beta diversity of salmonid gut microbiota, in line with the findings of the studies using other animals as subjects. As technical factors were most influential, it is important to harmonize them. Therefore, we suggest that these technical factors be standardized across studies to improve the comparability of microbiota findings. Otherwise, their effects should be taken into account when interpreting future study results.

5.1.2 Environmental influences on the gut microbiota

The environmental and host-associated factors across the 19 studies had a large impact on the alpha diversity of salmonid intestinal microbiota (Figure 4 and Figure 5). Among these biological factors, diet was ranked on the fourth place in explaining the variance of the beta diversity (Table 2) and this was visualized by the clustering of salmonid gut microbiota (Supplementary figure 1 in Paper I). Aside from the large significant effects on beta diversity, diet only had a significant and moderate impact on alpha diversity (Figure 4E). The alpha diversity of the insect-fed salmonids was numerically higher than the fish fed with commercial marine-based feeds (Figure 4E), which aligns with a previous meta-analysis on fish fed with black soldier fly larvae (*Hermetica illucens*) (Foysal & Gupta, 2022). Previous studies have suggested that the presence of chitin in diets containing insects leads to an enrichment of chitinase-producing bacteria that would not normally be present, hence increasing microbiota richness (Huyben et al., 2019). Alpha diversity of the samples collected from the fish fed plant-based diets was significantly lower than in all the other groups probably due to higher levels of antinutritional factors, such as phytate and saponins that may reduce microbial growth as previously reported (Reveco et al., 2014). Diet were reported to show similar effects on alpha and beta diversity in other non-salmonid fish species (Koh et al., 2016; Niu et al., 2020).

Interestingly, rearing system, water flow rate and daylight were the next most impactful factors on beta diversity after diet (Table 2). In

contrast to diet, these 3 factors showed large effects on alpha diversity rather than beta diversity (Figure 4). Daylight may be conflated with season and life-stage of the fish since young fry and fingerlings tend to receive continuous daylight while older broodstock may require shorter periods of daylight to prepare for breeding. Higher alpha diversity in recirculation systems (Figure 4D) was expected since the bacterial load in the water entering the rearing tanks of a recirculation system is much higher than that in a flow-through system (Attramadal et al., 2014). The higher hydraulic retention time without ozone or UV disinfection in the recirculation system results in a higher possibility that slow-growing microbes may stay longer and even grow after the initial disinfection (Deng et al., 2022; Vestrum et al., 2018). In addition, the maturity of the biofilter can play a role in modulating the microbiota in recirculation systems (Dahle et al., 2022). Water temperature is better controlled in recirculation systems, resulting in difference in the microbial community. However, the effect of temperature was one of the lowest factors influencing beta diversity in this meta-analysis (Table 2). This may be explained by the relatively low water temperatures salmonid fishes are typically reared compared to warm water fishes, e.g. tilapias and carps.

5.1.3 Host-associated influences on the gut microbiota

Among the host-associated factors, initial weight, which was largely correlated by the life stage or the age of the fish, had a smaller impact on beta diversity compared to the top five environmental and technical factors (Table 2), although it was very similar to the effect of diet. Notably, limited to only freshwater samples, the salmon before smoltification were younger and therefore smaller than the trout counterpart, thus different weight ranges were applied while translating weight values into categories (large salmon: not lower than 40 g, large trout: higher than 80 g) based on the general condition of the fish weights. The effect of life-stage (weight or age) has been found previously to be a more influential factor than location or rearing system and water temperature for wild Atlantic salmon and Chinook salmon (*O. tshawytscha*) (Llewellyn et al., 2016; Zhao et al., 2020). Previous studies on Atlantic salmon have also found that alpha diversity decreases as the fish ages due to their reduced ability to filter microbial communities as they mature (Heys et al., 2020; Huyben et al., 2020; Llewellyn et al., 2016).

5.2 Dietary filamentous fungi

5.2.1 Nutrient digestibility

Apparent digestibility of whole diets consistently showed higher ADC values in the reference diet compared with all fungi-supplemented diets, corroborating findings from similar research. Gaudhaman et al. (2025) reported comparable digestibility values and trends when using the same filamentous fungi (RO, RD, and AO). A notable difference between the studies was that ADC values for dry matter and crude protein were higher in our study, likely because of differences in faeces collection methods. Gaudhaman et al. (2025) used stripping to acquire the faecal material whereas in the present study collection belts were used to collect the faeces from effluent tank water. Several studies have illustrated that the collection method affects the overall digestibility (Hajen et al., 1993; Vandenberg & Noüe, 2001). Moreover, Gaudhaman et al. (2025) observed similar trends among diets, with AO exhibiting the highest digestibility, although there were slight variations in the ADC dynamics for RO and RD diets.

The higher ADCs of essential amino acids (EAA) relative to crude protein in the AO and RD diets suggest the presence of non-protein nitrogen sources. This observation aligns with the findings of Langeland et al. (2016), who reported similar results in their study on rainbow trout fed with RO. However, such a pattern was not observed for RO in the current study, which may be attributed to differences in fungi production methods between the studies.

At the ingredient level, the current study demonstrated the highest ADC for crude protein in the AO diet, followed by RO and RD. Conversely, Gaudhaman et al. (2025) reported the highest digestibility for crude protein in the RO diet, followed by AO and RD. The rationale behind these discrepancies is unclear but may be due to differences in experimental design, sampling methods, or environmental conditions.

5.2.2 Gut microbiota

The gut microbiota of fish fed the experimental diets were altered by the 30% replacement of conventional feed ingredients with filamentous fungal biomass, but the influence differed depending on the diet. The fish from the RO diet group had Shannon diversity values similar to the control counterpart (Figure 7A). However, according to the Bray-Curtis distance

plotted on the NMDS plot in Figure 7B, the samples in the RO group clustered away from the main cluster where the samples from the reference group and the other experimental groups scattered, indicating that the RO diet influenced the gut microbiota differently. As illustrated in Figure 6, fish fed the RO diet had uniformly different gut microbiota from the fish in the other group, dominated by *Bacillus* in most of the samples. As ubiquitous microbes in the natural environment, such as in dust, water, and feed ingredients, they could have been transferred via the feed. *Bacillus* species form endospores (Logan & Vos, 2015), rendering them highly resilient to different environments, which increases the likelihood of surviving heat and mechanical damage during feed production. Unfortunately, we did not analyse the microbiota in the feed or the ingredients and thus cannot confirm if the high relative abundance of *Bacillus* was introduced through the RO-feed. Once present in the feed, their environmental tolerance allows them to colonise the fish gut more effectively than many other bacteria. It is also possible that the RO diet shaped the fish gut into an environment advantageous for *Bacillus* species to grow and proliferate. Interestingly, aside from *Bacillus*, many of the bacteria that were significantly more abundant in the samples from the RO group are spore formers from phylum *Bacillota* (formerly *Firmicutes*) and *Spirochaetota*, including *Paenibacillus*, *Aneurinibacillus*, *Lysinibacillus*, and *Brevibacillus*. This could indicate that these spore formers benefit from the RO diet or the intestinal environment created in the gut from the RO diet. Another observation worth noting is that several genera with a significantly higher abundance in the gut of the fish fed with the RO diet have been associated with diseases and infection, such as *Paenibacillus* (Grady et al., 2016) and unassigned *Enterobacteriaceae* (Janda & Abbott, 2021), indicating a possible microbial disturbance by the RO diet. Considering that the RO diet had the lowest ADC values among the experimental diets (Tables 4 and 5), members of these disease and infection-related genera could potentially contribute to disturb the gut and result in lower digestibility.

The gut microbial composition of the fish fed the AO diet also differed from the Ref diet as well as the NI and RD group counterparts, although with large variation between the individuals. *Mycoplasma* dominated the gut of two fish fed the AO diet, whereas they only appeared as less abundant genera in the other groups. *Mycoplasma* have been found as a dominant part of the gut microbiota of rainbow trout and has been linked to many factors such as

gut sample type, fish health, weight, and species (Bozzi et al., 2021; Cheaib et al., 2020). Paper I also showed that *Mycoplasma* in rainbow trout of smaller size or reared at higher temperatures are more abundant (Cao et al., 2024). The high abundance of *Mycoplasma* observed in fish fed the AO diet may have also resulted from more mucus included in the sample collection, differences in fish size, or exposure to environmental sources such as water, feed, and air. *Weissella* had a significantly higher abundance in the gut of the fish fed the NI diet, whilst *Lactobacillus* were more enriched in the fish from the RD group, even though bacteria in these two genera were also found in other treatments. Previous studies have shown a probiotic potential of *Weissella* (Ahmed et al., 2022) and *Lactobacillus* (He et al., 2017), which indicates that the dietary addition of *N. intermedia* and *R. delemar* may benefit the hosts by shaping the fish gut in a way that favours the growth of the beneficial bacteria such as *Weissella* and *Lactobacillus*. A higher abundance of *Streptococcus* was found in relation to the reference diet. This genus was not present in any of the samples from the experimental diet groups. Certain *Streptococcus* species, such as *Streptococcus iniae*, are opportunistic fish pathogens (Madhusudhan et al., 2025; Weinstein et al., 1997), and the increased presence in the gut microbiota of the fish fed the Ref diet could potentially indicate a higher risk of infection and inflammation caused by the Ref diet or the gut environment shaped by the Ref diet.

In contrast to the RO group, the gut content of the fish fed the AO, NI, and RD diets exhibited the opposite pattern, with significance on alpha diversity but not on beta diversity indices. Apart from that, ANOVA confirmed the significant differences in the Shannon diversity between the fish fed the reference diet and the experimental diets AO, NI, and RD, but no significant difference was observed between the PD of the four experimental groups and the reference group. It is common to see different alpha diversities leading to diverse conclusions. The opposite results of the Shannon index and phylogenetic diversity indicate that the diets may not change the overall richness and evenness, but it can favour certain taxonomic lineages over others. The samples in the AO group clearly showed a much larger variety between individual fishes of both the beta diversities plotted compared with either the reference group samples or the other experimental diet counterparts. The significantly different bacterial composition of the samples from the AO group compared with the other groups may explain

this, which can also prevent differentially abundant genera from being detected by LDA.

5.3 Yeast probiotics

In the current study, we evaluated the potential of novel yeast species to be considered as probiotics and assessed their effects on gut health and immune responses in rainbow trout. By investigating new yeast strains, this research aimed to expand the range of functional probiotics available for aquaculture, thereby contributing to improved fish health and enhanced disease resistance. Yeasts are primarily considered immune stimulants, contributing to disease resistance by enhancing the host's innate immune response. Some yeast strains also produce metabolites that inhibit the growth of harmful bacteria in the gut. Together, these properties strengthen the host's defence mechanisms and reduce susceptibility to infectious diseases. Since innate immunity is a key component of early defence, we used *A. franciscana* as a model organism to select yeast strains that can confer protection against vibriosis under axenic conditions, and we evaluated the effectiveness of nine yeast strains in promoting disease resistance. Here, we also evaluated the optimal number of yeast cells required to confer effective protection against disease. Among all the tested strains and based on the selection criteria described in the method part, two yeast species were chosen for further study. The selected strains, RB and KM, were subsequently tested in rainbow trout to assess their potential probiotic behaviour and their effects on gut health and immune responses.

5.3.1 Gut microbiota

Relative abundance analysis of the gut digesta and feed samples (in Paper III) on the phylum level showed that *Firmicutes* dominated in both the feed and digesta, which has been found in previous studies. Glencross et al. (2025) found high abundance of *Firmicutes* and *Proteobacteria*, specifically *Bacillus, Paracoccus* and *Ralstonia spp.*, in diets for Atlantic salmon (B. Glencross et al., 2025). Despite its absence in feed, *Spirochaeotota* constituted 24.4% of the gut microbiota. The higher abundance of the *Spirochaeotota* originally residing in the fish gut may be favoured by the gut condition shaped by the CTL diet, as well as the RB and KM diets processed based on it. As many *Spirochaetota* are obligate anaerobes, it is possible that

these diets created a stable anaerobic environment for fermentation in the distal gut, which may have resulted from a maturing microbial ecosystem (Laursen et al., 2021). Thus, the relatively high abundance of *Spirochaeotota* in the gut microbiota of the fish fed the three experimental diets could be beneficial to the fish.

Analysis of the gut microbiota showed no significant effects of the RB and KM diets on either alpha or beta diversity indices at the middle and final sampling events (Figures in Paper III). Similar results have been reported in a study where Nile tilapia were exposed to aluminium and treated with a bacterial probiotic where both alpha and beta diversity remained unchanged between the control and probiotic groups (Yu et al., 2019). Apart from the aquaculture area, more research has been carried out on humans. In a study where healthy adults took a commercial probiotic containing *Bifidobacterium infantis* for 30 days, no significant changes were observed in gut composition or diversity (both α and β diversities) compared to placebo (Washburn et al., 2022). Another study in humans using a multi-strain probiotic fed for over 56 days have also shown no significant changes in Shannon and Simpson alpha diversities as well as Bray-Curtis beta diversity following probiotic intake (Rodenes-Gavidia et al., 2023). The commonly observed insignificant effect of probiotics on both alpha and beta diversity indices of the gut microbiota results from several causes. Gut microbial communities often have a highly stable core microbiota (Wuertz et al., 2021), which shows resistance to disturbance and resilience post-disturbance (Chen et al., 2021). Therefore, when the diet difference is not drastic enough, the gut microbiota can recover shortly after the disturbance. It is also impossible to capture all the functional changes only using alpha and beta diversity analyses, which is methodologically beyond their capability.

5.3.2 Gut health and Immunomodulatory effects

The addition of live yeast cells in the RB and KM diets not only influenced gut microbial composition but also modulated host gut barrier and immune responses. The *ocln* gene encodes occludin, a key tight-junction protein responsible for maintaining epithelial integrity (Hashizume et al., 2004). Previous studies have shown that *ocln* expression in fish gut tissues is sensitive to dietary factors, with upregulation linked to improved gut health and downregulation associated with dietary stress (Sagada et al., 2025; Su et

al., 2025). Similarly, *muc2*, which encodes mucin, a major component of intestinal mucus produced by goblet cells, is closely associated with innate immunity and pathogen resistance (Wang, Zhou, et al., 2024; Zhu et al., 2024). In the present study, the slight upregulation of *muc2* and significant increase in expression of *ocln* in RB diet and marked increase in KM diet during the mid-sampling period. This suggests enhanced production of occludin and mucin during the first phase of the trial. Over time, however, *muc2* returned to the basal level but *ocln* was downregulated.

In addition to barrier-related genes, immune-related markers also reflected the influence of dietary yeast. The *tlr2* gene, encoding Toll-like receptor 2, plays a central role in innate immune recognition of microbial components, triggering downstream cytokine responses including *il-1β*, *tnf-α*, and *tgf-β* (Mokhtar et al., 2023). While *il-1β* and *tnf-α* are pro-inflammatory cytokines associated with early immune activation, *tgf-β* functions as an immunoregulatory molecule that maintains immune balance (Chen et al., 2003; Reyes-Cerpa et al., 2012). *IL-1β,* is considered as pro-inflammatory cytokine that plays a key role in the immune system's response to infection or injury. In the current study, fish fed the RB diet showed upregulation of *tlr2, il-1β, and tnf-α* during the mid-sampling, indicating a transient pro-inflammatory state induced by the presence of live yeast. Concurrent upregulation of *tgf-β* likely reflected an effort to regulate excessive immune activation. By the end of the experiment, expression levels of pro-inflammatory genes had normalized, suggesting successful immune adaptation to the yeast-supplemented diet. A similar pattern was observed in fish fed the KM diet, where transient immune stimulation was followed by regulatory adjustments and partial normalization of gene expression toward the final sampling (Capaldo & Nusrat, 2009).

During the early immune response, proinflammatory cytokines like IL-1β and TNF-α are known to contribute to the disruption of tight-junction barriers, increasing epithelial permeability to allow immune cell infiltration and antigen sampling (Kaminsky et al., 2021). This disruption often involves mislocalization of *ocln* gene from the cell junctions, sometimes coupled with the upregulation of pore-forming claudins like claudin-2. Concurrently, these cytokines typically stimulate the expression and secretion of specific mucin-related genes as an initial defence mechanism (Capaldo et al., 2014; Kaminsky et al., 2021; Sanjabi et al., 2009). As inflammation resolves, anti-inflammatory cytokines and regulatory pathways, including transforming

growth *TGF-β*, become dominant to restore intestinal homeostasis. These regulatory signals help to maintain or restore barrier integrity by promoting epithelial cell repair, enhancing expression of certain sealing tight-junction proteins, and generally leading to a normalization or eventual downregulation of inflammation-associated genes to re-establish a healthy epithelial barrier. A similar expression pattern was observed in our study in response to RB and KM inclusion in the diet.

Overall, these findings indicate that while live yeast inclusion temporarily activates the intestinal immune system likely reflecting an initial host-microbe interaction, the response is subsequently balanced through regulatory mechanisms. The transient upregulation of immune and barrier-associated genes followed by normalization suggests that the yeast acted as a mild immune stimulant, enhancing gut readiness against pathogens without causing chronic inflammation. This controlled immune activation supports the proposed probiotic role of yeast in strengthening gut integrity, modulating immune homeostasis, and contributing to overall health in rainbow trout.

5.4 Mini meta-analysis of Paper I-II

A mini meta-analysis was performed to find associations between the results of Paper II and the findings in Paper I, as well as the previous experiments which had a similar experimental design. Similar to Paper I and the previous meta-analysis in shrimp (Cornejo-Granados et al., 2018), paper/study was also the most dominant factor that differentiated the microbial composition of the 246 samples from the four studies in Sweden (Figures 10-12, Table 8). These results indicated combined effects of multiple experimental factors in each study, including fish origin and early rearing conditions (discussed in 5.5.2), had the greatest impact on the gut microbiota. Interestingly, fish in Huyben et al. (2017) were from the Kälarne research station (Vattenbrukscentrum Norr AB, Kälarne, Sweden), where this experiment also was carried out. The fish in Huyben et al. (2018) and Huyben et al. (2019) were acquired from the same commercial producer (Vilstena fiskodling AB, Fjärdhundra, Sweden), whilst the fish in Paper II were from another producer (Älvdalslax AB, Älvdalen, Sweden). These three experiments were all performed in the Aquatic Facility at the Swedish University of Agricultural Sciences (SLU, Sweden). Despite these fish origin

and rearing differences, the gut microbiota of fish in Huyben et al. (2017) shared more similarities with fish in Paper II, whilst the fish from the same origin and rearing facility in Huyben et al. (2018) and Huyben et al. (2019) shared more similarities (Figures 10-12). However, both Huyben et al. (2017) and Paper II fed rainbow trout diets with high inclusions of fungi (12-30%) at 13 ºC, whereas the other studies had different temperatures (11 and 18ºC), live yeast and insect based diets (Huyben et al., 2018, 2019). These differences suggest that dietary inclusions of filamentous fungi and yeasts in inactivated forms may have a similar impact on the gut microbiota composition and diversities in rainbow trout (Figures 10-12). Similarly, Nyman et al. (2016) reported similar shifts in bacterial taxa (e.g., increased abundance of *Photobacterium* and *Lactobacillus*) when fishmeal was replaced by either filamentous fungus *A. oryzae* or yeast *S. cerevisiae* in diets fed to Arctic charr (*S. alpinus*) (Nyman et al., 2017). The similar impact of inactive filamentous fungi and yeasts in trout diets is possibly due to the common cell structure and components of fungi, such as β-glucans. β-glucans have been reported to have the ability to modify the gut microbiota of rainbow trout (Menanteau-Ledouble et al., 2022), and promote growth performance and immune responses in finfish (Doan et al., 2024). Recently, a review on postbiotics also showed that inactive fungi confer their health benefits mainly via the modulation of the gut microbiota, which may explain the similar effect of filamentous fungi and yeasts (Amobonye et al., 2025). However, more research is required to provide an extensive side-by-side comparison of the impact of different types of fungi in inactive form and the detailed mechanisms in salmonids.

Apart from the factor of paper/study, diet also significantly affected both alpha and beta diversities of the gut microbiota of the 246 fish across the four studies (Figures 11B, 12B). Compared with the fish on marine-based diets, the fish fed the yeast diets and filamentous fungal diets had significantly lower Shannon diversity indices. This is in line with the results of Paper I, though still unexpected, as the potential beneficial effects of the fungal diets should lead to higher alpha diversity (Huyben et al., 2018). The filamentous fungal diets were also found to be associated with higher abundance of *Weissella*, *Bacillus*, and *Lactobacillus* (Table 8 and Figure 10). Previous studies (Nyman, 2016; Singh et al., 2021) also reported higher abundance of *Bacillus* and *Lactobacillus* in fish fed filamentous fungal diets, while higher abundance of *Weissella* were found linked to plant-based diets (Ingerslev et

al., 2014) and plant-based diets supplemented with prebiotics (Lokesh et al., 2022) in the gut of rainbow trout. Lactic acid bacteria (LAB) and *Bacillus* species are largely considered beneficial as they play key roles in nutrient digestion and gut health (Chizhayeva et al., 2022; Jones et al., 2020; Kuebutornye et al., 2019; Nimalan et al., 2023), which indicates filamentous fungi in trout diets shaped the gut microbiota towards a beneficial direction. In contrast, fish fed the insect diets had a slightly higher Shannon diversity with no significant difference compared with the marine-based diet counterparts (Figure 11B), which also aligns with the results of a previous meta-analysis on fish fed black soldier fly larvae diets (*H. illucens*) (Foysal & Gupta, 2022). Moreover, the insect diets were related to higher abundance of *Pseudomonas*, *Oceanobacillus*, and *Corynebacterium* in Paper I (Table 8 and Figure 10). Higher abundance of *Pseudomonas* (Chen et al., 2024), *Oceanobacillus* (Busti et al., 2024), and *Corynebacterium* (Li et al., 2021) has been observed in the gut microbiota of fish fed insect diets compared with the control group counterparts in the individual studies, which indicates a microbial adaptation to chitin, lipids, or amino acid profiles of insect meals. Using Bray-Curtis distance NMDS plot, the fish fed the filamentous fungal and insect diets clearly separated from each other (Figure 12B), which could result from differences in fish origin and rearing conditions. It may also indicate that filamentous fungi and insects have different mechanisms for shifting gut microbiota, and therefore lead to different clusters in beta diversity. However, more research is required to investigate how filamentous fungi and insects interact with host gut microbiota in detail.

5.5 General considerations for future research

5.5.1 Early and legacy influences on the fish gut microbiota

In this thesis, I mainly focused on environmental factors that influence the gut microbiota of juvenile salmonids between 30-200 g in size, although more research needs to determine the influences on earlier life stages that impact the gut microbiota before experiments begin. In addition to the environmental factors in Paper I, previous studies have similarly reported that the gut microbial community in fish was shaped by factors related to their environment, such as host habitat (Kim et al., 2021; Sadeghi et al., 2023), rearing system (Deng et al., 2021), and surrounding water microbiota

(Ingerslev et al., 2014) at an early age. Deng et al. (2021) showed that early life exposure of larvae to biofloc, a heterogeneous aggregate of suspended particles and microorganisms, consistently increased the microbial interactions in the gut of juvenile Nile tilapia, with a legacy effect, where the first microbial colonization of the fish gut gradually disappeared after long periods of host development. Moreover, early rearing conditions, including vaccination (Andres et al., 2025), first feeding (Ingerslev et al., 2014), and probiotics application (Deng et al., 2022) also had significant impact on fish gut microbiota. Ingerslev et al. (2014) reported that the gut microbiota of rainbow trout changed separately according to the diet type after the first feeding, with increased abundance and diversity. After the first feeding, the microbiota was dominated by the phylum *Firmicutes* for plant-based fed fish while the phylum *Proteobacteria* dominated in the gut of marine fed fish (Ingerslev et al., 2014). Collectively, fish origin, rearing conditions, and treatments at an early age significantly influenced the initial inoculation of the fish gut microbiota before the experiments, which contributes to the variation of the gut microbial composition and diversity both at the beginning and end of each study (Figures 8-10). A comprehensive study focusing on the effects of the influencing factors on the gut microbiota of the fish at the very early life-stages, such as fry, would help to address this knowledge gap.

5.5.2 Optimizing fungal feed ingredients

In this thesis, filamentous fungi and yeasts were cultivated in bioreactors with little optimization, but it may be interesting to control the culturing environment, e.g., fermentation substrate, to control the cell morphology, such as cell size, for higher protein content or probiotic functions. Though no such study investigated the link between yeast probiotic size and the probiotic characteristics, research has been done on some strains of a popular bacterial probiotic genus, *Lactobacillus*. Previous studies have reported that long lactobacilli were often highly susceptible to acid and bile salts, and had higher antioxidant and aggregation activities compared with short lactobacilli counterparts, whereas short lactobacilli often had higher growth than their longer counterparts (Rajab et al., 2020). Moreover, Senz et al. (2015) also observed that short *Lactobacillus acidophilus* cells are more stable than long cells during industrial processing steps, such as freeze-drying, extrusion encapsulation, and storage following dried preparations. Fungal cells may not be as versatile as *Lactobacillus*, but it is still worth

considering whether a higher number of cells or larger fungal cells would be more effective in replacing protein or serving as a dietary supplement for rainbow trout.

The physiological status of fungal and yeast cells, whether live, inactive, autolysed, or sporulated, also plays a crucial role in determining their functional performance and sustainability when applied as single-cell proteins or probiotics in aquaculture systems. Despite previous studies on *N. intermedia* not finding a significant impact of feed preconditioning on shaping the gut microbiota of rainbow trout (Kokou et al., 2021), rainbow trout fed diets including autolyzed yeasts, *C. jadinii*, and *W. anomalus*, had significantly lower richness and diversity compared with the heat-inactivated counterparts (Agboola et al., 2023).

5.5.3 Long-term environmental impact and sustainability of fungi in feeds

Inclusion of fungi, such as single-cell proteins (SCPs) and probiotics, in aquafeeds offers significant environmental and sustainability benefits. Microbial biomass can be produced on organic waste substrates from low-cost agricultural residues, such as chicken manure, or industrial byproducts, such as organic wastes and industrial by-products (Karimi et al., 2018; Zha et al., 2021), that transform waste streams into valuable protein while reducing landfill use and greenhouse gas (GHG) emissions. Compared to conventional fishmeal and soy protein, fungal production requires less land, water, and energy, and generates a smaller carbon footprint. A recent study has reported that dietary inclusion of filamentous fungi *P. variotii* (PEKILO®) was negatively correlated with economic Fish in : Fish out ratio (eFIFO; a sustainability metric that improves upon the traditional FIFO ratio by using economic allocation to weigh the value of different marine ingredients) and significantly reduced food-competition feedstuff (FCF; ingredients used in animal feed that could otherwise be used for direct human consumption) in the diets (Hooft et al., 2025). Moreover, the inclusion of *P. variotii* also resulted in lower outputs of solid and dissolved wastes, such as solid phosphorus and dissolved nitrogen wastes (Hooft et al., 2025).

Use of probiotics in aquafeeds promotes fish health naturally and reduces the reliance on antibiotics that can lead to environmental contamination and antimicrobial resistance (Nathanailides et al., 2021). Moreover, probiotics in fish feeds also improve water quality of freshwater fish ponds and have the

potential to reduce toxic levels of ammonia and phosphorus (Mohammadi et al., 2020, 2021). These microbial solutions support circular bioeconomy principles by closing nutrient loops, lowering ecological impacts, and enhancing the long-term sustainability of aquaculture systems. However, some environmental challenges remain since fermentation of microbes requires large amounts of energy and infrastructure. Wastewater and byproducts from microbial fermentation also require management and processing. Moreover, potential contamination of the fermenters with mycotoxins, other microbes and genes that convey antimicrobial resistance should be monitored constantly to avoid feed safety problems. Advances in technology that increases the scale as well as efficiency will be key to further development and use of fungi in commercial aquafeeds, which is also true for fungi in livestock feeds and human food.

6. Conclusions and perspectives

Based on the results of Paper I, one can conclude that both biological and technical factors significantly influence alpha and beta diversity indices of salmonid gut microbiota. Among them, technical factors, such as paper/study, target hypervariable region, and DNA extraction kit, account for the greatest proportion of differences in gut microbiota composition. Moreover, technical factors heavily influenced the beta diversity and gut bacteria clustering, but their impact on alpha diversity was less strong, although the prevalence/dominance of specific bacteria could not be explained by a single factor. These results are consistent with previous research on humans and other animals which found that technical differences strongly impact beta diversity. In contrast, biological (non-technical) factors influenced alpha diversity to a greater extent than technical factors. The environmental factors led by diet impacted beta diversity and clustering of gut bacteria among the biological factors. Aside from that, host-associated factors only contributed to the variance of beta diversity and clustering of gut microbiota to a small extent and fish initial weight was the most dominant host-associated factor, which is also supported by previous studies. These findings demonstrate that technical methodologies must be standardised, and factors associated with host and environment must be accounted for in the experimental design of future studies.

Based on the results of Paper II, a 30% inclusion of the filamentous fungi, *A. oryzae*, *N. intermedia*, *R. delemar*, and *R. oryzae*, reduced nutrient digestibility and significantly altered the gut microbiota composition compared to the reference diet. Despite the short study period, these findings reveal that dietary inclusions of the tested filamentous fungi, *A. oryzae*, *N. intermedia*, *R. delemar*, and *R. oryzae*, have a rather strong impact on rainbow trout gut microbiota composition. The filamentous fungi evaluated in this study possess the potential to be promising protein sources for rainbow trout if nutrient digestibility can be improved in future diet formulations. Future research focusing on improving the nutrient digestibility of the filamentous fungi biomass with an extended duration is necessary to investigate the growth performance and nutrient digestibility of the fish fed the filamentous fungal diets across the long-term.

Based on the results of Paper III, both yeast species *R. babjevae* and *K. marxianus* help to maintain growth performance and alpha/beta diversity of

gut microbiota, but only *R. babjevae* significantly regulates the expression of immune-related genes, *oclna* and *tgf-β*. The reason that *K. marxianus* did not perform well as *R. babjevae* could be due to the lower viable yeast cell levels in the *K. marxianus* diet, but this requires further investigation. These findings demonstrate that *R. babjevae* have a higher potential as a probiotic in diets for rainbow trout, compared with *K. marxianus*. The probiotic effect of both *R. babjevae* and *K. marxianus* should be evaluated in future research with higher inclusion levels and an improved diet production method, and therefore higher viable yeast counts in the diets.

The results of this thesis underscore the need for further studies to understand the interactions between fungal-based diets and changes in the gut microbiota and intestinal function. Replacing fishmeal with filamentous fungi in aquafeeds remains a promising strategy to improve the sustainability of the aquaculture industry, but future research is still required to improve the nutrient digestibility of filamentous fungi in feeds for rainbow trout, as well as other species. Supplementation of yeast probiotics is a potential approach to modulate immune-related gene expression in the fish intestine, but there is room for improvement regarding the diet production and inclusion of more live yeast that survives in both feed and fish gut environments. Both lab and commercial scale experiments of extended periods are needed to confirm the significant effects of dietary fungi on fish growth in the long-term. The effects of fungal diets on intestinal histological changes should be investigated to link the shifts in gut microbiota in response to the functional diets. The effects of fungal diets on the expression of immune-related genes in other tissues, such as the liver, head kidney, spleen, and blood, at different time points should be assessed to elucidate the immune responses of fish fed fungal diets. Lastly, future research should identify bioactive components of the fungal inclusions and the detailed mechanisms involved to enable easier production of future functional diets. It will also be helpful to establish a quality control method, where different laboratories analyse a set of standard samples following the standard protocol to evaluate and compare their performance with a reference laboratory. The quality control results serve as an indication of accuracy and reliability for the samples of interest in the study, and thus the reproducibility and bias of diverse experimental settings can be evaluated.

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Popular science summary

Fish farming, or aquaculture, has become the fastest-growing food production sector in the world. As demand for seafood continues to rise, aquaculture plays an increasingly important role in ensuring that future generations have access to nutritious and sustainable sources of protein. However, this growth brings new challenges. To remain sustainable, aquaculture must find alternatives to traditional feed ingredients such as fishmeal and soy, which are expensive, environmentally demanding, and compete with food resources for humans and livestock.

A major key to overcoming these challenges may lie within the fish themselves — specifically, in the complex community of microorganisms that inhabit their intestines, known as the gut microbiota. These microbial populations are not just passive passengers; they actively influence digestion, nutrient absorption, metabolism, immune defence, and even behaviour. In other words, a healthy gut microbiota contributes to healthy, resilient fish. Understanding how different factors, especially diet, affect this internal ecosystem can help scientists design feeds that support both fish welfare and environmental sustainability.

This thesis investigated how biological, environmental, and dietary factors shape the gut microbiota of salmonid fish, focusing primarily on rainbow trout (*Oncorhynchus mykiss*), one of the most widely farmed freshwater species. It also explored the potential of microbial-based feed ingredients, such as fungi and yeasts, as innovative alternatives to conventional protein sources.

The first part of the research took a big-picture approach by combining data from many previously published studies on the gut microbiota of Atlantic salmon and rainbow trout. This meta-analysis revealed that the composition of gut microbes in salmonids is strongly affected not only by diet and environment but also by differences in experimental methods, such as how samples are collected and how DNA sequencing is performed. These findings highlight a crucial point: to compare results across studies and build reliable knowledge, the aquaculture research community needs more standardized approaches for sampling, sequencing, and data analysis.

The second study explored a novel use for filamentous fungi, microorganisms that can be cultivated on industrial by-products. Four fungal species (*Aspergillus oryzae*, *Neurospora intermedia*, *Rhizopus delemar*, and

Rhizopus oryzae) were grown on ethanol-production stillage, a leftover material from biofuel production, and then tested as potential protein sources in rainbow trout diets. Although these fungal ingredients were slightly less digestible than the standard control feed, they had a notable impact on the gut microbiota, increasing microbial diversity and potentially contributing to gut health. This suggests that with further optimization to improve digestibility, fungal biomass could become a valuable and sustainable feed ingredient that also helps recycle industrial waste streams.

In the third study, the focus shifted to probiotics, live microorganisms that can provide health benefits to the host. Two yeast species, *Kluyveromyces marxianus* and *Rhodosporidium babjevae*, were tested as dietary supplements in rainbow trout. The results showed that *R. babjevae* in particular promoted beneficial bacterial groups in the intestine and influenced the expression of immune-related genes. These effects suggest that this yeast could strengthen the fish's natural defences and improve overall health, offering a promising addition to future aquaculture feeds.

Together, the findings from these studies deepen our understanding of how fish feed composition interacts with the intestinal microbiota and fish physiology. They demonstrate that microbial-based ingredients, whether in the form of fungi used as feed proteins or yeasts used as probiotics, can contribute to more sustainable, health-promoting aquaculture systems.

By harnessing the power of beneficial microbes and repurposing waste materials into valuable feed components, this research supports a shift toward circular and environmentally responsible aquaculture. Ultimately, it brings us one step closer to feeding the world's growing population without compromising the health of our ecosystems or the fish that sustain them.

Populärvetenskaplig sammanfattning

Vattenbruk, eller akvakultur, är idag den snabbast växande livsmedelssektorn i världen. I takt med att efterfrågan på fisk och skaldjur ökar spelar vattenbruk en allt viktigare roll för att säkra framtidens livsmedelsförsörjning. Samtidigt står branschen inför stora utmaningar. För att vara hållbart måste vattenbruket minska sitt beroende av traditionella foderingredienser som fiskmjöl och soja, vilka både är kostsamma och belastar miljön samt konkurrerar med livsmedelsproduktion för människor och boskap.

En viktig del av lösningen kan finnas inuti fisken själv, i den komplexa gemenskapen av mikroorganismer som lever i tarmen, det så kallade tarmmikrobiomet. Dessa mikrober är inte bara passiva passagerare; de spelar en aktiv roll i matsmältning, näringsupptag, ämnesomsättning och immunförsvar. Ett friskt mikrobiom bidrar till en frisk och motståndskraftig fisk. Genom att förstå hur olika faktorer, särskilt fodrets sammansättning, påverkar tarmens mikrobiota kan vi utveckla foder som främjar både fiskens hälsa och miljömässig hållbarhet.

Denna avhandling undersökte hur biologiska, miljömässiga och framför allt kostrelaterade faktorer påverkar tarmmikrobiotan hos laxartade fiskar, med särskilt fokus på regnbåge (*Oncorhynchus mykiss*). Arbetet utvärderade även potentialen hos mikrobiella foderingredienser, såsom svampar och jäst, som innovativa och hållbara alternativ till konventionella proteinkällor.

I den första studien gjordes en meta-analys där data från tidigare publicerade studier på Atlantlax och regnbåge kombinerades. Resultaten visade att sammansättningen av fiskens tarmmikrobiota påverkas starkt inte bara av diet och miljö, utan även av skillnader i forskningsmetoder, till exempel hur prover tas och hur DNA-sekvensering genomförs. Detta understryker behovet av standardiserade metoder inom forskningen för att kunna jämföra resultat och bygga tillförlitlig kunskap.

Den andra studien undersökte möjligheterna att använda trådsvampar som nya proteinkällor i fiskfoder. Fyra svamparter, *Aspergillus oryzae*, *Neurospora intermedia*, *Rhizopus delemar* och *Rhizopus oryzae*, odlades på restprodukter från bioetanolframställning och testades som foderingredienser i regnbågsdieter. Trots att dessa svampingredienser hade något lägre smältbarhet än kontrollfodret påverkade de tarmens mikrobiella mångfald positivt, vilket tyder på att de kan vara lovande och hållbara

alternativ, särskilt om deras näringsmässiga egenskaper förbättras ytterligare.

I den tredje studien låg fokus på probiotika, levande mikroorganismer som kan ha hälsofrämjande effekter. Två jästarter, *Kluyveromyces marxianus* och *Rhodosporidium babjevae*, testades som fodertillskott för regnbåge. Resultaten visade att särskilt *R. babjevae* ökade förekomsten av gynnsamma bakteriegrupper i tarmen och påverkade uttrycket av immunrelaterade gener, vilket tyder på att den kan stärka fiskens immunförsvar och bidra till bättre hälsa.

Sammantaget bidrar avhandlingens resultat till en djupare förståelse av hur fodrets sammansättning och mikrobiella tillskott påverkar tarmens ekosystem och fiskens fysiologi. Studierna visar att mikrobiella ingredienser, både svampar som proteinkällor och jäst som probiotika, kan spela en viktig roll i utvecklingen av mer hållbara och hälsofrämjande foder för vattenbruket.

Genom att utnyttja nyttiga mikroorganismer och omvandla restprodukter till värdefulla foderråvaror stödjer detta arbete utvecklingen mot ett cirkulärt och miljömässigt ansvarsfullt vattenbruk. På så sätt tar vi ett steg närmare en framtid där vi kan försörja en växande befolkning utan att kompromissa med fiskhälsa eller ekosystemens balans.

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REVIEW

REVIEWS IN Aquaculture

A meta-analysis revealing the technical, environmental, and host-associated factors that shape the gut microbiota of Atlantic salmon and rainbow trout

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Abstract

Salmonids, specifically Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*), are commonly farmed and their gut microbiota plays important roles for optimal growth, health, and physiology. However, differences in experimental design, technical factors and bioinformatics make it challenging to compare the results from different studies and draw general conclusions about their influence on the fish gut microbiota. For a more comprehensive understanding of the gut microbiota, we collected all the publicly accessible 16S rRNA gene sequencing data with clearly stated sample metadata from freshwater Atlantic salmon and rainbow trout intestinal contents and mucosa sequenced on the Illumina MiSeq platform. A total of 783 samples from 19 published studies were included in this meta-analysis to test the impact of the technical, environmental, and host-accociated factors. This meta-analysis revealed that all the tested factors significantly influenced the alpha and beta diversities of the gut microbiota of salmon and trout. Technical factors, especially target region and DNA extraction kit, affected the beta diversity to a larger extent, while host-associated and environmental factors, especially diet and initial fish weight, had a higher impact on the alpha diversity. Salmon had a higher alpha diversity and higher abundance of *Enterococcus* and *Staphylococcus* than trout, which had higher abundance of *Weissella* and *Mycoplasma*. The results of this meta-analysis fill in a critical knowledge gap that demonstrate technical methodologies must be standardized and factors associated with host and environment need to be accounted for in the future design of salmonid gut microbiota experiments.

KEYWORDS
16S rRNA, fresh water, gut bacteria, microbiome, salmonid

1 | INTRODUCTION

The microbiota describes the collection of the microorganisms, such as bacteria, fungi, protozoa, and viruses, living in a certain environment. Microbes play key roles in the development and maintenance of different physiological functions of their eukaryotic host, in humans as well as other animals. The microbiota residing in the gut (gastrointestinal tract) within animals contributes to host nutrient

absorption,[1] metabolism,[2] aging,[3] immune system regulation,[4] and protection against pathogen invasion.[5] Meanwhile, the microbial communities are also constantly influenced by host factors, such as developmental stage,[6] health conditions,[7] and species,[8] as well as environmental factors, including temperature,[9] light,[10] and diet.[11]

In aquaculture, gut microbiota is important to aquatic animals due to their beneficial effects, especially the production of essential nutrients, for example, short chain fatty acids and vitamins. Studies have shown that intestinal microbiota are significantly affected by changes in environmental (abiotic) and host (biotic) factors, and therefore impair or promote their growth performance and health under different conditions.[12,13] Moreover, studies have shown impacts of environmental factors, including dietary composition, feed ration, temperature, rearing systems or habitat, as well as host-associated factors, including fish taxa, age, growth rates, and health status, on the gut microbiota of cultured fish in a laboratory setting as well as in the wild.[8,12–24] However, fish microbiota studies typically only investigate one or two factors per study, while controlling for several others, and it is difficult to compare studies with wide ranges in the factors mentioned above, in addition to differences in technical factors, for example, DNA extraction, PCR, and bioinformatic methods.

Salmonid fishes, particularly Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*), are two of the most commonly farmed fish species and are economically important to the global aquaculture industry.[25] However, there is a lack of research on the influence of technical, environmental, and host-associated factors on the salmonid gut microbiota, and the evaluated factors are often study specific. In this context, a meta-analysis, as a potent systematic method to re-analyse and summarize the results collected from multiple individual studies in a specific field,[26] can be applied to generalize the results of previous studies and give insights on future research within the area. To the best of our knowledge, there is no systematic meta-analysis reviewing studies focusing on the relations between salmonid gut microbiota and the potential influence of the three kinds of factors.[26]

The primary objective of this study was to perform a systematic meta-analysis on the freshwater salmonid studies to determine the effect size and rank of each technical, environmental and host-associated factors that influence the gut microbiota, specifically the alpha and beta diversities. The secondary aim was to correlate individual gut microbes with groups of these associated factors. We used the QIIME2 pipeline and SILVA 138 database to analyse 16S rRNA gene sequences from 19 studies composed of 783 samples from the gut of freshwater Atlantic salmon and rainbow trout.

2 | METHODS

2.1 | Systematic literature search

For the selection and collection of raw 16S rRNA sequence data for the meta-analysis in this study, all peer-reviewed published papers related to 'salmonid gut microbiota' were identified and then manually checked to ensure the suitability for the meta-analysis (Figure 1). The potential studies of interest were filtered by Title–Abstract–Keyword search on SCOPUS using 18 keyword combinations (['salmon', 'trout' or 'char'], ['gut' or 'intestine'], and ['microbiome', 'microbiota' or 'microbe']) and Title/Abstract search of PubMed database using the same combinations. The combined search from these two databases resulted in 229 full-text research articles published from 1 January 2011 to 31 December 2022.

Due to the low number of studies using salmonid species other than Atlantic salmon and rainbow trout, only these two species were selected for the meta-analysis (Figure 1). In addition, data collection was limited to in vivo studies sampling intestinal digesta or mucosa (rather than the whole intestinal tissue) from healthy (not obviously diseased or infected) non-triploid freshwater salmonids (Atlantic salmon before smoltification and freshwater-raised rainbow trout) without selection for any purposes in the meta-analysis to reduce the complexity and generalize the results for future research in this area. Moreover, only studies using Illumina MiSeq 16S rRNA gene sequencing were chosen because it was the most common sequencing platform. After that, 50 studies were checked for data accessibility due to the necessity of both clearly stated sample metadata and raw 16S rRNA gene sequencing data required to perform the meta-analysis. As a result, 19 studies meeting the selection criteria (Table 1) were identified for further processing and meta-analysis. All the raw 16S rRNA gene sequencing data and sample metadata were downloaded from NCBI Sequence Read Archive (SRA; https://www.ncbi.nlm.nih.gov/sra).

All the factors that potentially affected the gut microbiota were compiled and categorized for all the studies while evaluating them no matter if they were specifically addressed in the original study. After the final 19 studies (Table 1) were selected, only the 15 factors clearly stated in more than half of the studies (at least 10 studies) were analysed in this meta-analysis.

2.1.1 | 16S rRNA gene sequence data processing

All raw 16S rRNA sequence data generated by the Illumina MiSeq platform were processed by the next-generation microbiome bioinformatics platform QIIME2[42] (https://qiime2.org/) version 2022.2 following the tutorials provided by the QIIME2 team (https://docs.qiime2.org/2021.11/tutorials/). Raw sequences were imported into QIIME2, demultiplexed, end-joined, and denoised with chimera removal using QIIME2 built-in DADA2 method. To include most samples without compromising the quality of the data, the sequences were trimmed to maintain a minimum quality score of Q25. Samples with <2000 reads and taxa with fewer than 10 reads in that individual study were discarded to focus on higher abundant taxa in the meta-analysis. After that, the samples without enough replicates ($n < 5$) or proper control groups were also excluded. Then the sequences were taxonomically classified using the classifier pre-trained by RESCRIPT[43] on the full-length 16S rRNA gene SILVA v138 database[44] with a 99% confidence provided by QIIME2 (https://docs.qiime2.org/2023.5/

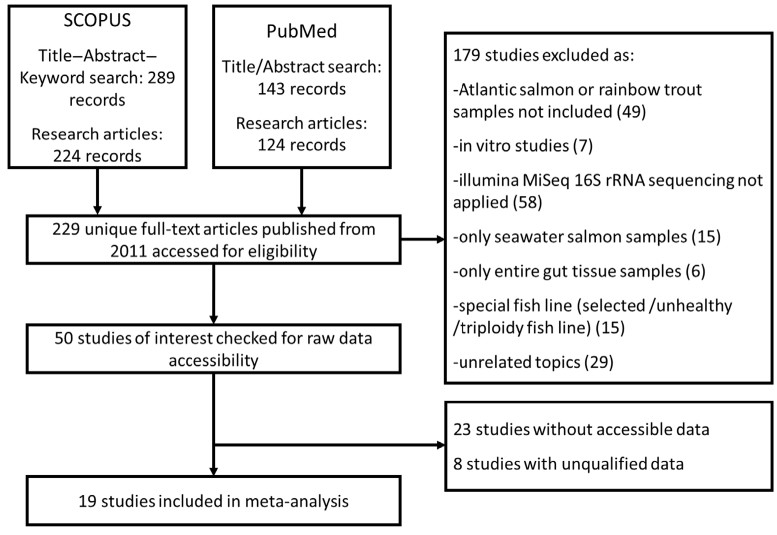


FIGURE 1 Flow chart illustrating the systematic literature search and data selection processes. The values in brackets indicate the total number of studies excluded next to each sub-criteria used.

data-resources/#public-microbiome-data). Classified sequences were taxonomically filtered to remove mitochondria, chloroplast, Archaea, and Eukaryotes. After that, all the pre-processed sequence and sample metadata were merged using QIIME2 merge commands for the following combined processing.

2.1.2 | Combined sequence data processing across studies

A phylogenetic tree was generated by QIIME2 built-in fast tree command using the merged data. The merged files were then piped to R[45] version 4.2.0 for further analysis. The data were cleaned and then filtered to discard the Amplicon Sequence Variants (ASVs) unclassified on phylum level. or with a prevalence lower than 3 throughout the whole dataset including all the samples using R tidyverse[46] ver1.3.1, stringr[47] ver1.4.0, and dplyr[48] ver1.0.9 packages. A phyloseq[49] (version 1.40.0) object was built and then all the samples were rarefied to 2838 sequences (the lowest number of sequences over 2000 sequences in one sample) to reduce the influence of sampling depths. The taxa were agglomerated on genus level for beta diversity analysis as not all the ASVs were classified on the lower level.

2.2 | Meta-analysis

Alpha diversity indexes and beta diversity distance matrixes were generated by R picante[50] ver1.8.2 and R vegan[51] ver2.6-2 and then visualized via ggplot2[52] ver3.3.6 and ggpubr[53] ver0.4.0 packages. Shapiro–Wilk normality tests[54] were used to evaluate the normality of the distribution of alpha diversity values, whereas non-parametric Kruskal–Wallis tests[55] were used to evaluate significance of the influencing factors on alpha diversity. Post hoc tests were done by Dunn tests[56] using Benjamini–Hochberg method[57] to determine pairwise differences between the groups. Faith phylogenetic diversity values were also regressed in generalized linear mixed-effects models by R lme4[58] and car[59] packages to analyse the contribution of the factors. Permutational Multivariate Analysis of Variance (PERMANOVA)[60] (9999 permutations were performed) with weighted Unifrac distance were used to evaluate significance of the influencing factors on beta diversity. The *p*-values <0.05 were considered as significant. Pairwise PERMANOVA was performed on all the factors with more than two subgroups to learn the differences between every two subgroups. Multivariate homogeneity of groups dispersions was tested by PERMDISP[61] in which both ANOVA (distances to centroids were calculated) and permutational analysis (999 permutations) were performed. Linear discriminant analysis Effect Size (LEfSe)[62] analysis results were generated by R microbiomeMarker[63] ver1.2.2 to determine the differentially abundant genera associated with a specific subgroup of a factor. Kruskal–Wallis cutoff of 0.05 and Linear Discriminate Analysis (LDA) cutoff of 2 were applied. The *p*-values were corrected by Benjamini–Hochberg method[57] to account for multiple comparisons, and q values lower than 0.05 were regarded as significant. All the results were visualized by ggplot2[52] and ggpubr[53] packages.

A total of 15 factors were analysed in this meta-analysis: paper/study, species, fish initial weight (large salmon/LS: ≥40 g; small salmon/SS: <40 g; large trout/LT: >80 g; small trout/ST: ≤80 g),

TABLE 1 Studies of interest with accessible data used for the meta-analysis on the freshwater salmonid gut microbiota.

Reference	Country	Number of samples[a]	Species	Target hypervariable region	Intestinal region	Rearing system	DNA extraction kit	Accession number
Leeper et al.[14]	Iceland	54/54	*Salmo salar*	V3–V4	Proximal and distal intestine	NA	QIAamp PowerFecal Pro	PRJNA732903
Bruni et al.[27]	Italy	10/11	*Oncorhynchus mykiss*	V3–V4	Proximal intestine	Flow-through	QIAamp Fast Stool Mini	PRJNA703401
Rimoldi et al.[28]	Italy	35/36	*Oncorhynchus mykiss*	V3–V4	Proximal and distal intestine	Flow-through	DNeasy PowerSoil	PRJEB28677
Weththasinghe et al.[29]	Norway	77/78	*Salmo salar*	V3–V4	Distal intestine	Recirculation	QIAamp Fast Stool Mini	PRJNA762510
Terova et al.[30]	Italy	12/12	*Oncorhynchus mykiss*	V3–V4	Proximal and distal intestine	Flow-through	DNeasy PowerSoil	PRJEB38845
Terova et al.[31]	Italy	24/24	*Oncorhynchus mykiss*	V3–V4	Proximal and distal intestine	Flow-through	DNeasy PowerSoil	PRJEB28677
Webster et al.[32]	UK	48/48	*Salmo salar*	V3–V4	Proximal and distal intestine	Flow-through and wild[b]	DNeasy PowerSoil	PRJEB30953
Bugten et al.[15]	Norway	38/60	*Salmo salar*	V4	Distal intestine	Recirculation	QIAamp DNA Mini	PRJEB48548
Huyben et al.[33]	Sweden	63/72	*Oncorhynchus mykiss*	V4	Distal intestine	Flow-through	QIAamp DNA Mini	PRJNA351922
Huyben et al.[34]	Canada	10/10	*Oncorhynchus mykiss*	V3–V4	Distal intestine	Flow-through	QIAamp Fast Stool Mini	PRJNA767341
Rudi et al.[35]	Norway	38/40	*Salmo salar*	V3–V4	Distal intestine	NA	LGC Mag Midi	PRJNA413667
Wang et al.[36]	Norway	16/16	*Salmo salar*	V1–V2	Distal intestine	NA	QIAamp Fast Stool Mini	PRJNA660116
Li et al.[37]	Norway	103/103	*Salmo salar*	V1–V2	Distal intestine	NA	QIAamp Fast Stool Mini	PRJNA730696
Krogdahl et al.[38]	Norway	27/27	*Salmo salar*	V1–V2	Distal intestine	Recirculation	QIAamp Fast Stool Mini	PRJNA539907
Huyben et al.[39]	Sweden	46/46	*Oncorhynchus mykiss*	V4	Distal intestine	Flow-through	QIAamp Fast Stool Mini	PRJNA454155
Huyben et al.[16]	Sweden	95/96	*Oncorhynchus mykiss*	V4	Distal intestine	Flow-through	QIAamp Fast Stool Mini	PRJNA408116
Biasato et al.[40]	Italy	12/12	*Oncorhynchus mykiss*	V4	Proximal and distal intestine	Flow-through	DNeasy PowerSoil	PRJEB51166
Hines et al.[41]	USA	15/40	*Oncorhynchus mykiss*	V4	Proximal and distal intestine	Recirculation	DNeasy PowerSoil	PRJNA750741
Baumgartner et al.[17]	UK	60/60	*Salmo salar*	V1–V2	Distal intestine	Recirculation	QIAamp DNA Mini	PRJNA800661

[a]The number before the slash indicated the number of samples that were included in the meta-analysis that passed quality filtering, while the number after the slash was the original number of gut samples collected.
[b]It is a trans-location study in which half of the wild and farmed fish were transferred to a farmed or wild environment while the other half were kept in the same environment as before. All the listed DNA extraction kits except LGC Mag Midi were manufactured by the global provider Qiagen.
Abbreviation: NA, not available.

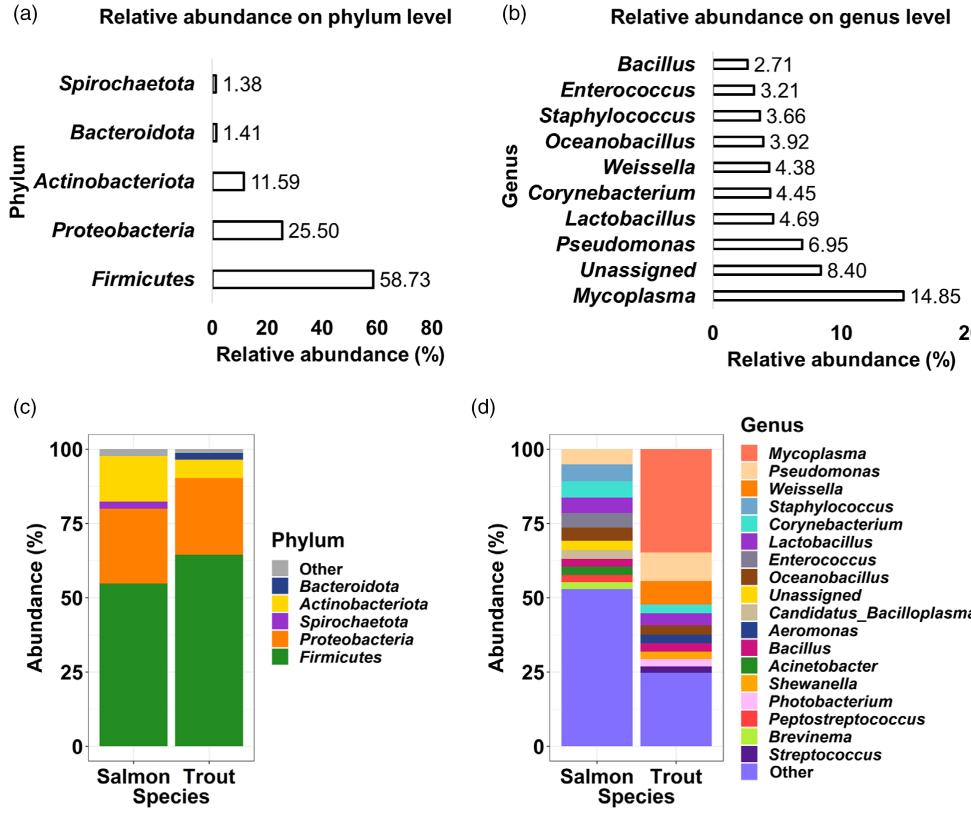


FIGURE 2 (A–D) Relative abundance of 783 gut microbiota samples from 19 freshwater salmonid studies. (A,C) phylum level. (B, D) genus level. Only the phyla more abundant than 1% and the genera with abundance values higher than 2% are shown in all four plots. The values beside the bars in plot A and B show the relative abundance of the corresponding phylum or genus. The *unassigned* is a collection of the genera unclassified on genus level from all the phyla.

specific growth rate (SGR; high: >1.2, otherwise considered as low), feed conversion ratio (FCR; high: >2, otherwise considered as low), weight gain (high: >140 g, otherwise considered as low), rearing system, daylight, temperature (high: >15°C, low: <13°C, other temperatures considered as mid), water flow rate (high: >8 L/min, otherwise considered as low), diet, target hypervariable region, intestinal segment, DNA extraction kit, and DNA polymerase.

3 | RESULTS

3.1 | Systematic literature search

Among all the 229 unique full-text studies after the combined search we excluded 49 studies using species other than Atlantic salmon and rainbow trout, 7 in vitro studies, 58 studies using sequencing platforms other than Illumina MiSeq, 15 seawater studies, 6 studies that only collected entire gut tissue samples, 15 studies using special fishlines, and 29 studies focusing on non-relevant topics. As a result, 50 studies were further checked for data accessibility. Among these, 23 studies were excluded for the absence of clearly labelled raw sequence data even after requesting assistance from the authors. Another eight studies including only sequence data of low quality (i.e., <Q25) or abundance (i.e., <2000 sequences) after processing by the uniform method described in the methods section. Only 19 studies met our requirements (Tables 1 and S1) and were included in the meta-analysis. The filtered studies included 783 samples that were represented by 7190 ASVs across 554 genera and 23 phyla.

Among all the samples, 96.8% were from Europe, while only 3.2% of the samples were collected in North America. Among the European countries, Northern European countries including Norway (38.2%), Sweden (26.1%), and Iceland (6.9%) provided 71.1% of all the samples. Exactly 58.9% of the samples were unsmoltified freshwater Atlantic salmon while 41.1% were rainbow trout. A total of 73.2% of all the

TABLE 2 The impact of the influencing factors on the alpha diversity of gut microbiota in freshwater salmonid fishes using Faith phylogenetic diversity.

Factor	Factor type	Sample size	*p*-value	Chi-squared
Paper/study	Mixed	783	<0.0001	609.08
Species	Host-associated	783	<0.0001	375.10
Initial weight	Host-associated	706	<0.0001	369.36
Rearing system	Environmental	572	<0.0001	357.53
Flow rate	Environmental	406	<0.0001	220.93
FCR	Host-associated	315	<0.0001	209.05
Target hypervariable region	Technical	783	<0.0001	208.17
Daylight	Environmental	549	<0.0001	205.48
SGR	Host-associated	356	<0.0001	192.67
Weight gain	Host-associated	193	<0.0001	135.44
Diet	Environmental	745	<0.0001	132.63
DNA extraction kit	Technical	783	<0.0001	127.98
Temperature	Environmental	680	<0.0001	126.48
Intestinal region	Host-associated	783	<0.0001	114.35
DNA polymerase	Technical	713	<0.0001	55.76

Note: The *p*-values and chi-squared values were generated from Kruskal–Wallis tests.
Abbreviations: FCR, feed conversion ratio; SGR, specific growth rate.

samples were distal intestine samples, whereas 25.5% were from both proximal and distal intestine and 1.3% were from the proximal intestine. As for the target hypervariable regions, 39.3% of the samples targeted V3–V4 region, followed by 34.4% of them targeting V4, while samples targeting region V1–V2 consisted of 26.3% of all the samples.

3.2 | General microbiota characteristics

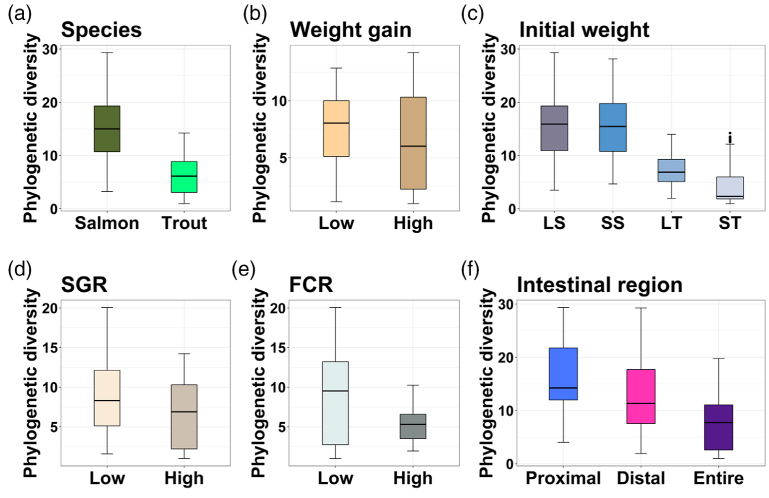
Among the 23 phyla present in the samples, three phyla dominated the salmonid gut microbiota with a total relative abundance of over 95.8% (Figure 2). Firmicutes contributed about 58.7% of the abundance as the most abundant phylum, followed by Proteobacteria that accounted for ~25.5% and Actinobacteria consisted of around 11.6% of the abundance. The alpha diversity indices were calculated after the ASVs were rarefied to 2838, which reduced the library size differences among the samples from different studies to facilitate alpha diversity comparisons.

Shapiro–Wilks test indicated Faith phylogenetic diversity (Faith PD) of the samples was not normally distributed ($W = 0.966$, $p < 0.001$), so non-parametric Kruskal–Wallis tests were performed to identify the significance of the factors. According to the Kruskal–Wallis tests, all 15 factors had significant effects on Faith PD (Table 2). Intestinal microbiota in Atlantic salmon had significantly higher Faith PD than the rainbow trout counterpart ($p < 0.001$, chi-squared = 375.1; Table 2 and Figure 3a). Furthermore, initial weight did not differentiate the Faith PD of either Atlantic salmon subgroups or rainbow trout subgroups significantly even though separated the species ($p < 0.001$, chi-squared = 369.4; Table 2 and Figure 3c). Regarding rearing systems, the recirculating system showed significantly higher Faith PD compared with both wild and flow-through counterparts, while the flow-through system had the lowest alpha diversity ($p < 0.001$, chi-squared = 357.5; Table 2 and Figure 4d). The samples collected from the mid-temperature ($p < 0.001$, chi-squared = 126.5; Table 2 and Figure 4b; Data S1) or high water flow rates ($p < 0.001$, chi-squared = 220.9; Table 2 and Figure 4c) had significantly higher Faith PD values than the others. As for the target hypervariable regions, primers targeting V1–V2 allowed for significantly higher phylogenetic diversity than V3–V4 and V4, whereas V4 alone gave the lowest phylogenetic alpha diversity ($p < 0.001$, chi-squared = 208.2; Table 2 and Figure 5b; Data S1).

In addition, Faith PD values were analysed by generalized linear mixed-effects models. In the best-fit model with the lowest Bayesian Information Criterion (BIC) value,[64] factor paper/study was considered as a random effect variable, while all other factors as fixed effect factors. The factors with low significance ($p > 0.05$) were removed one by one to find the factors making big impacts on Faith PD values. After the factors were selected, the interactions among the factors were also investigated. According to the ANOVA results (Table 4 and Data S1), species (chi-squared = 6.57, *p*-value = 0.01) and intestinal region (chi-squared = 8.19, *p*-value = 0.02) had significant influence on Faith PD, whereas all the other factors in the model were not significant.

Similar to alpha diversity, PERMANOVA also showed significant influences of all 15 factors on weighted UniFrac distances. Apart from that, group dispersions of all the factors except for factor species showed significant inhomogeneities in betadisper results (Figure S1).

The PERMANOVA of weighted UniFrac distances revealed a significant association between the microbiota of the gut samples and all



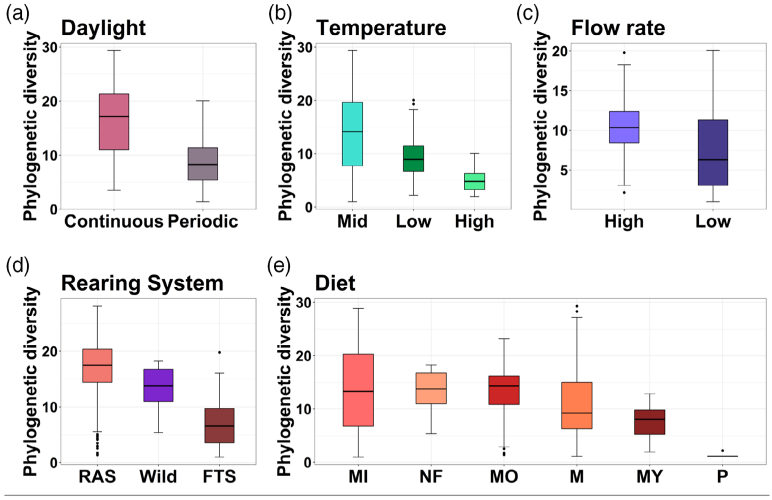
Tag	Factor	*p* value	Chi squared	Tag	Factor	*p* value	Chi squared
A	Species	<0.0001	375.1	D	SGR	<0.0001	192.7
B	Weight gain	<0.0001	135.4	E	FCR	<0.0001	209.0
C	Initial weight	<0.0001	369.4	F	Intestinal region	<0.0001	114.4

FIGURE 3 (A) Boxplots of Faith phylogenetic (alpha) diversity of 783 gut microbiota samples from 19 freshwater salmonid studies (host-associated factors). (B) Weight gains higher than 140 g are regarded as high, otherwise considered as low. (C) 'LS' stands for large salmon weighing at least 40 g while small salmon weighing lower than 40 g are labelled as 'SS'. 'LT' stands for large trout weighing more than 80 g while small trout weighing not more than 80 g are labelled as 'SS'. (D) specific growth rates higher than 1.2 are considered as high, otherwise regarded as low. (E,F) Feed conversion ratios higher than 2.0 are considered as high, otherwise considered as low. The table below the plots provides the *p*-values and chi-squared values from Kruskal–Wallis tests.

the technical factors including scientific paper/study, target hypervariable region, DNA extraction kit, and DNA polymerase. Technical factors explained most of the variation in data with significant inhomogeneities. Paper/study was the dominant factor explaining over 60% of the variance of the beta diversity (Table 3), which is more than twice the value for the primer target hypervariable region in the second place. In the weighted UniFrac beta diversity PCoA plot (Figure 6), PC1 explained 52.3% of the variation and the data points from each study were clustered together with half the studies overlapping while the other half clustered separately. Similarly, the target hypervariable region was also an important driver of the clustering of the intestinal microbiota (Figure 7f).However, in the best-fit generalized linear mixed-effects model, no technical factors was included (Table 4). In order to reveal which taxa that was most associated with the different technical factors, LEfSe at 0.05 significance level was applied. The LEfSe identified diverse microbial genera associated with different target hypervariable regions (Figure 8). High *Lactobacillus* abundance was significantly associated with region V1-V2 (Figure 8f and Table S2), while an enrichment of *Staphylococcus* instead was related to the V3–V4 region. In the samples targeting region V4, *Mycoplasma*, *Pseudomonas*, and *Weissella* were significantly more abundant.

3.3 | Environmental influence on salmonid gut microbiota

Among the other factors, the environmental factors explained more variance of the beta diversity than the host-associated factors. The most explanatory environmental factor was diet accounting for over 18.6% of the total beta diversity variance, while the rearing system, water flow rate, daylight, and the intestinal region explained around 11.6%–15.2% of the beta diversity variance (Table 3). Several abundant genera were identified using LEfSe that were associated with different dietary types. *Mycoplasma* was associated with plant-based diet, whereas the fish without extra feed supply related to high abundance of *Candidatus_Bacilloplasma* and *Brevinema* (Table S2). Marine-based feeds with different substitutes or supplements were also related to diverse bacterial groups. *Enterococcus*, *Weissella* and *Staphylococcus*, and *Pseudomonas* were enriched in the samples fed marine-based feeds with inclusion of insects, yeasts, and other ingredients not originally in the feeds, respectively (Table S2). The second most explanatory environmental factor was rearing system (Table 3 and Figure 7b) that showed recirculating aquaculture (RAS) and flow through systems (FTS) as separate clusters but with some overlap. Moreover, diet, temperature, and rearing system were also included in



Tag	Factor	*p* value	Chi squared	Tag	Factor	*p* value	Chi squared
A	Daylight	<0.0001	205.5	D	Rearing system	<0.0001	357.5
B	Temperature	<0.0001	126.5	E	Diet	<0.0001	132.6
C	Flow rate	<0.0001	220.9				

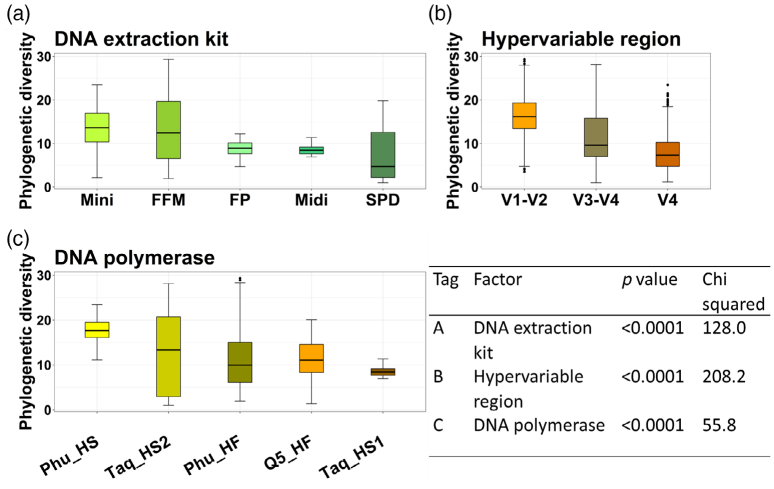
FIGURE 4 (A) Boxplots of Faith phylogenetic diversity of 783 gut microbiota samples from 19 freshwater salmonid studies (environmental factors). (B) Temperatures lower than 13°C are considered as low temperature, while 'High' indicates temperatures higher than 15°C. All other temperatures are considered as mid temperatures. (C) Water flow rates higher than 8 L/min were categorized as high, otherwise considered as low. (D) 'RAS' stands for recirculating aquacultural system, while 'wild' indicate a wild-like environment, and 'FTS' is the flow-through system. (E) 'M' stands for marine-based feeds. 'MI', 'MY', and 'MO' indicate marine-based feeds with inclusions of insects, yeasts, and other nutrient sources such as other prebiotics or oils. 'P' indicates plant-based feeds, whereas 'NF' indicates a wild-like environment without any feed provided. The table below the plots provides the *p*-values and chi-squared values from Kruskal–Wallis tests.

the best-fit model, though without any significance (Table 4). ANOVA (Data S1) showed that no feed diet (co-efficient = 2.06, *t*-value = 1.84) and mid-temperature (co-efficient = 2.78, *t*-value = 0.94) had higher impacts on Faith PD. Several abundant genera were identified using LEfSe that were associated with different rearing systems. *Mycoplasma*, *Weissella*, and *Pseudomonas* were enriched in the gut samples collected from the FTS, whereas *Bacillus* and *Enterococcus* were significantly more abundant in RAS (Figure 8d). There are also some genera related to the wild environment, such as *Candidatus_Bacilloplasma*, *Aeromonas*, *Brevinema*, and unassigned genera in *Enterobacterales* (Figure 8d).

Most of the abundant genera associated with diet and rearing system were also related to other environmental influencing factors including water flow rate (Figure 8e), daylight (Table S2), intestinal region (Table S2), and water temperature (Figure 8b). Unlike diet and rearing system, these factors only explained <14.2% of the beta diversity variance (Table 3). *Mycoplasma* and *Pseudomonas* were abundant in fish living in low flow rate water, while *Weissella*, *Candidatus_Bacilloplasma*, *Lactobacillus*, *Brevinema*, and *Streptococcus* were associated with high water flow rate (Figure 8e). *Staphylococcus*, *Enterococcus*, and *Oceanobacillus* were related to continuous daylight, whereas *Weissella* and *Pseudomonas* were abundant in the fish living in environments with periodic light (Table S2). *Pseudomonas* was found associated with the distal intestine, while *Mycoplasma* and *Candidatus_Bacilloplasma* were abundant in the entire gut samples (Table S2). Many genera were related to the proximal intestine, such as *Oceanobacillus*, *Lactobacillus*, *Phyllobacterium*, and *Enterococcus* (Table S2). High temperature was associated with *Mycoplasma and Pseudomonas*, whereas *Staphylococcus* were abundant in the samples collected from the fish living in environments of the low temperatures below 13°C (Figure 8b).

3.4 | Host-associated influence on salmonid gut microbiota

Host-associated factors, such as initial weight and species, only had minor influences on beta diversity. The most explanatory host-associated factor was initial weight and only accounted for 16.0% of the variation, whereas the other host factors (i.e., SGR, FCR, species, and weight gain) explained <11.0% of the beta diversity variation (Table 3). However, host-associated factors species (chi-squared = 6.57, *p*-value = 0.01; Table 4) and intestinal region (chi-squared = 8.19, *p*-value = 0.02; Table 4) had significant influence on



Tag	Factor	*p* value	Chi squared
A	DNA extraction kit	<0.0001	128.0
B	Hypervariable region	<0.0001	208.2
C	DNA polymerase	<0.0001	55.8

FIGURE 5 Boxplots of Faith phylogenetic diversity of 783 gut microbiota samples from 19 freshwater salmonid studies (technical factors). (A,B) 'Mini' and 'Midi' indicate the products designed for the extraction of microbial DNA from small amounts of samples. 'FFM' stands for the DNA fast extraction kit designed for small amounts of faecal samples, while 'FP' indicates the method designed for faecal samples with a built-in bead-beating step. 'SPD' stands for the DNA extraction kit designed for soil samples with a built-in bead-beating step and an addition of DNase. (C) 'Q5', 'Phu', and 'Taq' indicate Q5, Phusion, and Taq DNA polymerases followed by 'HS' or 'HF' which stands for hot start or high-fidelity characteristics. The numbers only show products from different companies. The table beside the plots provides the *p*-values and chi-squared values from Kruskal–Wallis tests.

TABLE 3 The impact of the influencing factors on the beta diversity of gut microbiota in freshwater salmonid fishes using weighted UniFrac and PERMANOVA.

Factor	Factor type	Sample size	*p* value	*R* squared	Variance explained (%)	Pseudo-*F*
Paper/study	Mixed	783	<0.001	0.618	61.8	68.57
Target hypervariable region	Technical	783	<0.001	0.244	24.4	125.90
DNA extraction kit	Technical	783	<0.001	0.191	19.1	46.00
Diet	Environmental	745	<0.001	0.187	18.7	29.75
DNA polymerase	Technical	713	<0.001	0.173	17.3	32.46
Initial weight	Host-associated	706	<0.001	0.160	16.0	37.02
Rearing system	Environmental	572	<0.001	0.152	15.2	46.41
Flow rate	Environmental	406	<0.001	0.141	14.1	64.26
Daylight	Environmental	549	<0.001	0.123	12.3	54.48
Intestinal region	Host-associated	783	<0.001	0.116	11.6	51.26
SGR	Host-associated	356	<0.001	0.110	11.0	48.10
FCR	Host-associated	315	<0.001	0.090	9.0	38.16
Species	Host-associated	783	<0.001	0.081	8.1	68.45
Temperature	Environmental	680	<0.001	0.075	7.5	20.98
Weight gain	Host-associated	193	<0.001	0.061	6.1	25.51

Note: 9999 permutations were performed in each of the PERMANOVA tests to obtain the *p* value, *R* square, and pseudo-*F* values.
Abbreviations: FCR, feed conversion ratio; SGR, specific growth rate.

Faith PD. Moreover, trout showed the highest impact among all the subgroups, with a co-efficient of −8.05 and *t*-value of −2.56 (Data S1), followed by both proximal and distal intestinal region (co-efficient = −5.97, *t*-value = −2.17; Data S1). Large salmon was associated with *Lactobacillus*, *Oceanobacillus*, and *Corynebacterium*, while *Staphylococcus* was abundant in small salmon (Table S2). In contrast to

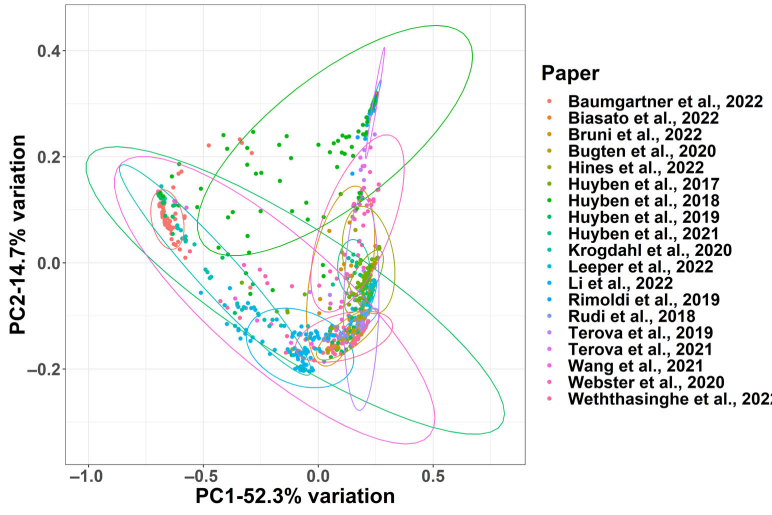


FIGURE 6 Principal coordinate analysis (PCoA) plot of weighted UniFrac distance of 783 gut microbiota samples from 19 freshwater salmonid studies (beta diversity). The studies are represented by different colours. Circles represent 95% confidence intervals.

salmon, different bacterial groups were found enriched in trout. Large trout were associated with *Pseudomonas* and *Weissella*, whereas *Mycoplasma* and *Shewanella* were abundant in small trout. For host-associated factors including specific growth rate (SGR), feed conversion ratio (FCR), and weight gain (WG), only a few genera were found to differ in abundance among the subgroups. *Shewanella* and *Mycoplasma* were associated with high SGR, while *Pseudomonas* was abundant in low SGR fish guts (Table S2). *Mycoplasma*, *Pseudomonas*, and *Aeromonas* were related to high FCR, while *Weissella* were identified as enriched groups associated with low FCR (Table S2). High WG was associated with *Shewanella* and *Mycoplasma*, while *Pseudomonas*, *Oceanobacillus*, and *Corynebacterium* were abundant in the fish with low WG (Table S2). Species only explained <8.1% of the variance but drove the separate clustering of gut microbiota between Atlantic salmon and rainbow trout in two directions in the PCoA plot (Table 3 and Figure 7a). As for the abundant microbial genera associated with these two species, *Mycoplasma* and *Weissella* were highly related to rainbow trout, while *Staphylococcus* and *Enterococcus* were associated with Atlantic salmon samples (Figure 8a).

4 | DISCUSSION

In this meta-analysis, we comprehensively collected all the available 16S rRNA gene sequence data from Atlantic salmon and rainbow trout from the literature, and then we filtered the data based on predefined criteria, e.g. high quality, well labelled and available data. The selected data were re-analysed using a standard set of parameters and the same bioinformatics tools were used for all the studies to minimize the bias arising from the experimental and analytical procedures. Our aim was to determine the contribution of technical, environmental, and host-associated factors that influenced the gut microbiota of salmonid fishes. In all the 783 samples from 19 studies, a dominant presence of Firmicutes, Proteobacteria and Actinobacteria on the phyla level indicated a core microbiota (Figure 2), which has been identified as dominant phyla in other salmonid (Salmonidae family) and ray-finned (Sparidae family) fish species, for example, Arctic charr[11] and gilthead sea bream, respectively.[65] In contrast, the gut microbiota of cyprinid (Cyprinidae family) fish species has been dominated more by Proteobacteria than Firmicutes, such as in Nile tilapia,[66,67] or dominated by Fusobacteria, for example, in common carp.[68,69] The differences in the core microbiota between genetic families of fish species are highly related to the host conditions (e.g. genetic and physiological divergences), the disparate environment they live in (e.g. water microbiota),[70] as well as the long-playing co-evolution between the host species/genus/family and their gut microbiota.[71]

Our meta-analysis indicated that paper/study is the overall most dominant factor that affects both the alpha and beta diversity of gut microbiota in freshwater salmonids (Table 3 and Figure 6). In addition, all the factors that were evaluated in this meta-analysis had a significant effect, which was similar to previously reported factors in the meta-analysis on the microbiota of shrimp.[72] A meta-analysis on the gut microbiota of 1046 healthy humans from around the world found that most factors influenced the beta diversity similar to the present study, specifically the environment (e.g. diet and housing) explained up to 20% of the variation whereas host effects (e.g. ancestry) had minor affects.[73] Aside from the paper/study, technical factors including target hypervariable region and DNA extraction kit were also dominant factors in shaping the beta diversity of

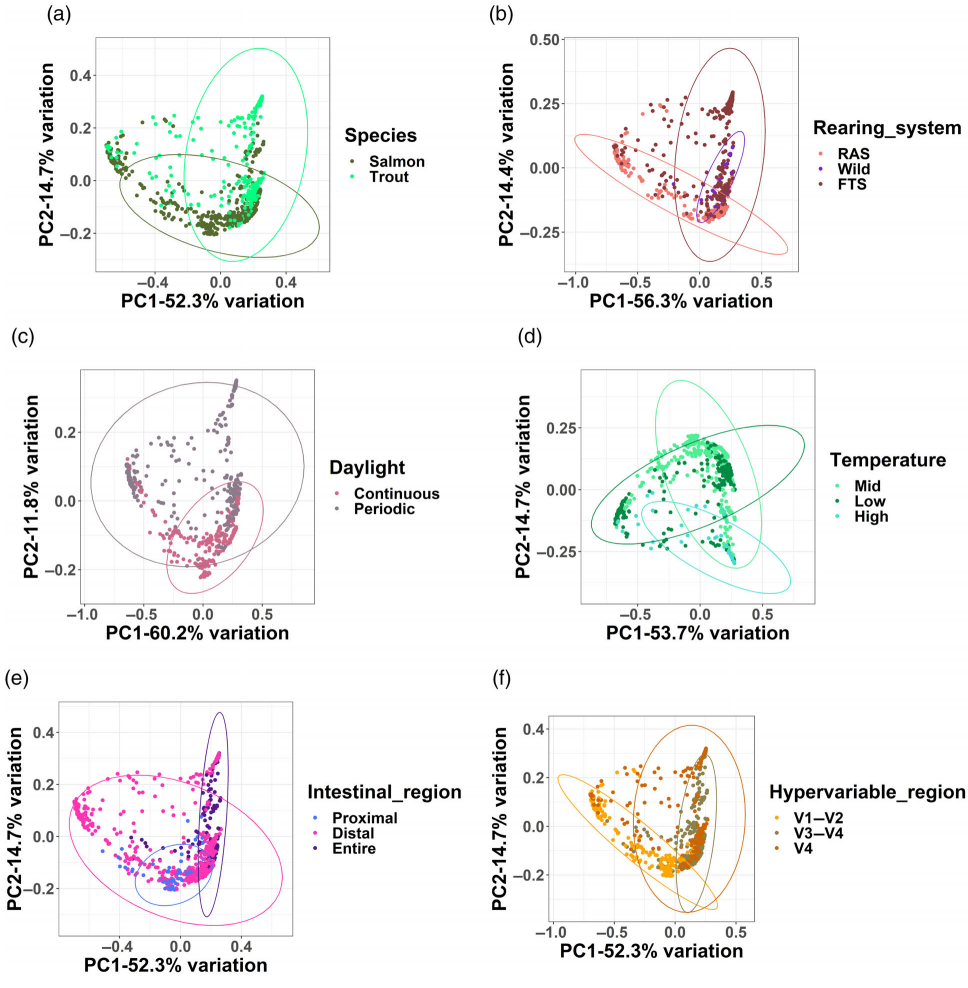


FIGURE 7 (A–F) Principal coordinate analysis (PCoA) plots of weighted UniFrac distances of 783 gut microbiota samples from 19 freshwater salmonid studies (beta diversity). Samples grouped by the host-associated factor (species), environmental factors (rearing system, daylight, water temperature, and sampled intestinal region), and technical factors (target hypervariable region). Circles represents 95% confidence intervals. 'RAS' stands for recirculating aquaculture system, while 'Wild' indicates a wild-like environment, and 'FTS' stands for flow-through system.

salmonid gut microbiota, but not in shaping the alpha diversity counterpart (Figures 5 and 7; Tables 2 and 3). In the studies testing the effects of target hypervariable regions and DNA extraction kits using the stool samples from humans and mice, significant shifts of microbiota composition related to experimental conditions were also found.[74] In addition, DNA extraction kits have been found to change the microbial compositions in faeces between zebrafish, horses, dogs, cats, and mice.[75] Hart et al.[75] suggested that the size of zebrafish and their intestine compared with the faecal biomass could change the yield of microbial DNA extracted using commercial kits since smaller intestines would have a higher proportion of host compared to bacterial DNA. These authors also mentioned that the fibre content in the diet, time post feeding and digestive enzymes could change the amount of faecal biomass and consequently the amount of DNA to be extracted. The variation in the amount of microbial DNA could bias the amplification efficiency of either target hypervariable region during PCR and sequencing of 16S rDNA. Therefore, the DNA extraction kit and target region would influence more of which microbes are identified rather than their diversity. In contrast, a study on human faeces found that DNA extraction method had little effect on microbial communities, while the target region had an immense impact.[76] The results shown in the present study revealed that both DNA

TABLE 4 The significance of the top influencing factors in the best-fit generalized linear mixed-effects model.

Factor	Chi-squared	*d.f.*	*p*-value
Species	6.57	1	0.01
Intestinal region	8.19	2	0.02
Diet	7.24	6	0.30
Temperature	2.17	3	0.54
Weight gain	0.22	2	0.90
Rearing system	0.19	2	0.91
SGR	0.01	2	1.00

Note: Type 3 ANOVA was performed to obtain the *p*-values and chi-squared values.
Abbreviations: d.f.: degree of freedom; SGR, specific growth rate.

extraction kit and target region had a high impact on beta diversity of salmonid gut microbiota, in line with the findings of the studies on other animals. Therefore, we suggest that these technical factors should be standardized across studies in order to improve comparisons of the microbiota between studies. If not, the influence of these technical factors should be considered when comparing the results from future studies.

The environmental and host-associated factors across the 19 studies had a large impact on the alpha diversity of salmonid gut microbiota (Figures 4 and 5). Among these factors, the environmental factor of diet ranked fourth place in explaining the variance of the beta diversity (Table 3) and this was visualized by the clustering of salmonid gut microbiota per diet (Figure S1). Aside from the large significant effects on beta diversity, diet only had a significant and moderate impact on alpha diversity (Figure 4e). The alpha diversity of the insect-fed salmonids was numerically higher than the fish fed with commercial marine-based feeds (Figure 4e), which aligns with a previous meta-analysis on fish fed with black soldier fly larvae (*Hermetica illucens*).[77] Previous studies have suggested that the presence of chitin in diets containing insects leads to an enrichment of chitinase-producing bacteria that would not normally be present, hence increasing microbiota richness.[39] Yeast based diets have been found to have beneficial effects on the gut health and microbiota of rainbow trout,[16] so it was unexpected to see the low level of alpha diversity (Figure 4e). Alpha diversity of the samples collected from the fish fed plant-based diets was significantly lower than in all the other groups, probably due to higher levels of antinutritional factors, such as phytate and saponins, that reduce microbial growth as previously reported.[78] Diet has also had similar effects on alpha and beta diversity in other non-salmonid fish species.[79,80]

Interestingly, environmental factors of rearing system, water flow rate and daylight were the next most impactful factors on beta diversity after diet (Table 3). In contrast to diet, these three factors showed large effects on alpha diversity rather than beta diversity (Figure 4). Daylight may be conflated with season and life-stage of the fish since young fry and fingerlings tend to receive continuous daylight while older broodstock may require shorter periods of daylight to stimulate breeding events. Higher alpha diversity in recirculation systems (Figure 4d) was expected since the bacterial load in the water entering the rearing tanks of recirculation systems is much higher than the counterpart in flow-through systems.[81] The higher hydraulic retention time without ozone or UV disinfection in the recirculation system results in a higher possibility that slow-growing microbes may stay longer and even grow after the initial disinfection.[21,82] In addition, the maturity of the biofilter can play a role in modulating the microbiota in recirculation systems.[83] Water temperature is better controlled in recirculation systems, resulting in different microbial communities compared to flow-through systems vulnerable to seasonal changes in temperature. However, the effect of temperature was one of the lowest factors influencing beta diversity in this meta-analysis (Table 3). This may be explained by the relatively low water temperatures salmonid fishes are typically reared compared with warm water fishes, for example, tilapias and carps.

Among the host-associated factors, initial weight, which was largely correlated by the life stage or the age of the fish, had a smaller impact on beta diversity compared with the top five factors that were environmental or technical (Table 3), although it was very similar to the effect of diet. Notably, limited to only freshwater samples, the salmon before smoltification were younger and therefore smaller than the trout counterpart, thus different weight ranges were applied while translating weight values into categories (large salmon: not lower than 40 g, large trout: higher than 80 g) based on the general condition of the fish weights. The effect of life-stage (weight or age) has been found previously to be a more influential factor than location or rearing system and water temperature for wild Atlantic salmon and Chinook salmon (*Oncorhynchus tshawytscha*).[22,84] Previous studies on Atlantic salmon have also found that alpha diversity decreases as the fish ages due to their reduced ability to filter microbial communities as they mature.[65,84,85]

In the best-fit generalized linear mixed model, all seven fixed effects were either host-associated or environmental factors, which is also in line with our finding that environmental factors have larger impacts on alpha diversity while technical factors impact beta diversity to a larger extent (Tables 2 and 3). Among the seven fixed effects, host-associated factors of species and intestinal region showed significant effects on shaping Faith PD of fish gut microbiota. It was expected that the factor of species has a significant influence on alpha diversity as previously reported,[86] and the statistics on Faith PD (Table 2) also supported this. However, according to the Kruskal–Wallis tests (Table 2), the intestinal region had the lowest chi-squared value among all the host-associated and environmental factors. The difference may be caused by the other factors in the model, such as random effect of paper/study or other fixed effects. Moreover, interactions among the factors were also investigated. All possible interaction effects between the seven fixed effects were tested, but no significant interactions were found. That did not mean these factors did not interact with each other, it is still possible that there are significant interactions between the factors not included in the best-fit model, or that the interactions were hidden by the simple linear regression. Regarding beta diversity analysis, significant

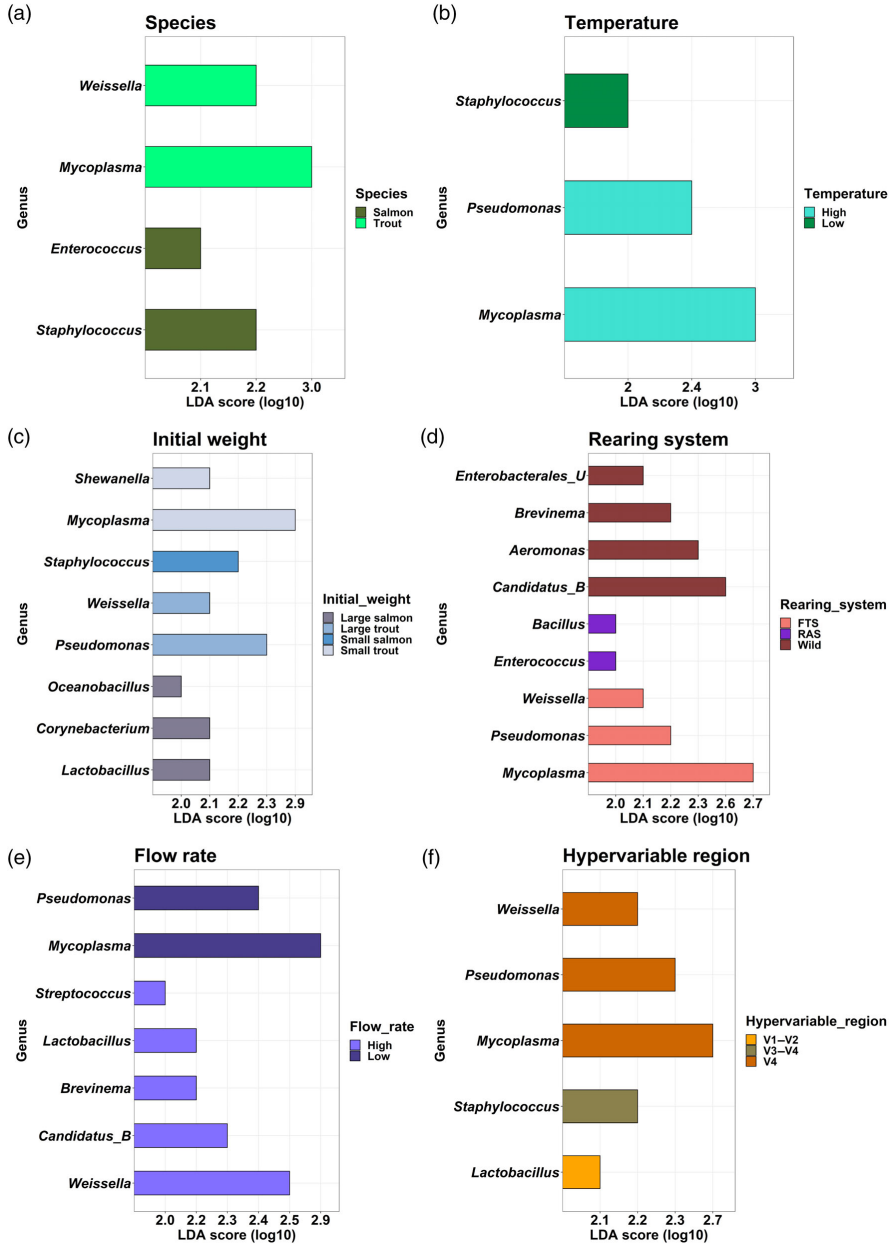


FIGURE 8 (A–F) Linear discriminant analysis Effect Size (LEfSe) results of abundant genera for 783 gut microbiota samples from 19 freshwater salmonid studies. The *y*-axis shows the enriched genera associated with different subgroups while the *x*-axis indicates the linear discriminant analysis (LDA) score (log10). High levels of classification were used for the genera not classified on lower levels (labelled with a *_U* suffix). Only the genera with an LDA score no lower than 2.0 are presented in the plots. The *q* values adjusted by Benjamini–Hochberg method lower than 0.05 are considered significant. '*Candidatus_B*' is short for '*Candidatus_Bacilloplasma*'.

heterogeneities of the beta diversity data were observed except for the factor of species, which may be a reason why all the factors showed significant influences on beta diversity. PERMANOVA was used due to its superior statistical power and insensitivity to heterogeneity in dispersions of comparisons with Analysis of similarities (ANOSIM) and the Mantel test,[87] although it is still possible that the PERMANOVA results we found were correlated between factors to some extent.

Regardless of the influencing factors, on the genus level, a core microbiota of *Mycoplasma*, *Pseudomonas*, *Lactobacillus*, *Corynebacterium*, *Weissella*, *Oceanobacillus*, *Staphylococcus*, and *Enterococcus* was found in the gut of Atlantic salmon and rainbow trout (Figure 2). Although, *Mycoplasma* and *Pseudomonas* were either found in very high abundance in half the studies and very low abundance in the other half, while the other genera were found more consistently (Figure 2). Previous studies have reported that these eight dominate genera in this meta-analysis are common in other freshwater fish species, such as grass carp (*Ctenopharyngodon idellus*)[88] and zebrafish (*Danio rerio*).[89] The dominant genus *Mycoplasma* accounted for over 14.8% abundance and was commonly found in both faeces and mucosa in the salmonid gut. A higher load of *Mycoplasma* in diseased Atlantic salmon[90] has made *Mycoplasma* a proxy for poor salmonid health, while its function is still not clear. A recent study has found that *Mycoplasma* produces the essential amino acid arginine[91] and may not only be associated with disease. *Lactobacillus* and *Weissella* were widely distributed across the gut microbiota of salmonids and these are usually considered as beneficial microorganisms due to their probiotic functions,[92,93] yet some of them have been reported as pathogens in salmonids.[94,95] Many species belonging to *Pseudomonas*, *Corynebacterium*, *Staphylococcus*, and *Enterococcus* are widely considered multidrug-resistant pathogens and opportunistic pathogens in fish as well as other animals,[96–99] and thus may be responsible for some differences in fish performance. *Oceanobacillus* is a relatively rare genus in freshwater fish species and little knowledge has been gained about them except their presence in fish. *Oceanobacillus* species are commonly distributed in seawater, but have also been found in a wastewater treatment system[100] and on rainbow trout skin.[101] A previous study performed on Beluga sturgeon (*Huso huso*)[102] indicated that fish feed with a higher inclusion of fishmeal resulted in gut microbiota more enriched in *Oceanobacillus*, therefore the fishmeal inclusion in marine-based diets may be responsible for the high abundance of this genus.

This meta-analysis for the first time presents a systematic re-analysis of all freshwater salmonid gut microbiota studies that provided 16S rRNA gene raw sequencing data generated by the Illumina MiSeq platform, but with some improvable limitations. At the very beginning of the meta-analysis, all possible influential factors were included, however, only the factors clearly indicated in at least half of the studies remained in the final analysis to generate meaningful results summarized across at least 10 out of the 19 studies. As a result, some factors of interest and importance, such as the pH and dissolved oxygen of the water, feeding frequency and fish density, were discarded. The lack of information on all these factors in the 19 studies resulted in their exclusion, while they may have significant impacts on the fish gut microbiota. Another limitation is the complexity of the influencing factors that overlap with each other, thus future improvement in the design of meta-analyses need to be performed. However, it is possible to control confounding effects by statistical analysis[103] or bioinformatics handling given adequate data[104] and the information about how each factor is generated. Apart from that, many studies of interest were also excluded before the final analysis due to inaccessibility of the authors and the raw data with clearly stated sample metadata. It would be very beneficial to promote publications with open access data and to include as much information as possible in future studies, which allows more secondary studies to compare and re-analyze the data to answer future questions. Only samples sequenced by the Illumina MiSeq platform were studied in this meta-analysis due to its high use in previous studies of interest and to avoid more complexity in analysing the results. Other more powerful Illumina sequencing platforms, such as HiSeq, and NovaSeq, provide higher resolutions and coverages, but also require higher computing capacity and longer time to process and analyse. Apart from Illumina, longer regions, such as full length 16S rRNA (all nine regions), can be sequenced on using Oxford Nanopore, Pacific Biosciences, Element Biosciences and other platforms to get more accurate representation of microbial compositions of the samples down to the species and strain level. Thus, a more in-depth systematic review should be performed in the future when there are sufficient sequence data and metadata on the gut microbiota of salmonid fishes, especially with future advancements in DNA extraction methodologies, sequencing technologies, microbial sequence databases, bioinformatic and meta-analysis tools.

5 | CONCLUSIONS

Overall, our findings indicate that all the factors mentioned in this study significantly influenced alpha and beta diversity indices of salmonid gut microbiota. PERMANOVA revealed that technical factors, such as paper/study, target hypervariable region and DNA extraction, heavily influenced the beta diversity and clustering of gut bacteria, whereas their impact on alpha diversity was not as strong. Paper/study was expected to be the most influential since the combination of different kinds of factors are combined for each individual study. Previous studies on humans and livestock animals agreed with our meta-analysis on salmonids that found target hypervariable region and DNA extraction kit highly impact gut microbiota results. Compared with the technical factors, host-associated and environmental factors influenced alpha diversity to a larger extent. Also, some of them, such as diet and initial weight, are much more explanatory than others in influencing beta diversity. The environmental factors led by diet impacted the beta diversity and clustering of gut bacteria among the host-associated and environmental factors. Aside from that, host-associated factors only contributed to the variance of beta diversity and clustering of gut bacteria to a minimal extent and fish initial weight was the most dominant host-associated factor, which was again supported by previous studies. These findings show three types

of factors influence the gut microbiota of salmonids, which further demonstrate that technical methodologies must be standardized and factors associated with host and environment need to be accounted for in the experimental design of future studies.

AUTHOR CONTRIBUTIONS

David Huyben: Conceptualization; supervision; writing – review and editing. **Shuowen Cao:** Writing – original draft; formal analysis. **Johan Dicksved:** Writing – review and editing; supervision. **Torbjörn Lundh:** Supervision; writing – review and editing; project administration. **Aleksandar Vidakovic:** Writing – review and editing; supervision. **Parisa Norouzitallab:** Supervision; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

All raw sequencing data analysed in this study are publically available on the NCBI SRA website under the accession numbers mentioned in Table 1. Data generated by this meta-analysis are available upon reasonable request.

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This thesis investigated the influence of host-associated, environmental, and technical factors on the gut microbiota of freshwater salmonids. It also evaluated filamentous fungi as alternative protein sources and yeast probiotics in diets for rainbow trout. Results revealed significance of all the tested factors significantly influenced the alpha and beta diversities of the gut microbiota in Atlantic salmon and rainbow trout. It also indicates the potential of filamentous fungi as alternative protein sources and yeast probiotics in diets for rainbow trout.

Shuowen Cao has received her graduate education at the Department of Applied Animal Science and welfare, Swedish University of Agricultural Sciences (SLU) in Uppsala, Sweden. She received her Master of Science degree in Biology at Uppsala University (UU), Uppsala, Sweden.

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