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Alternative feed sources

Effects on gut microbiota, immunity and health of rainbow trout

Aprajita Singh

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Abstract

Shifting from conventional fishmeal- and soymeal-based aquafeed to low-cost, sustainable dietary alternatives is essential for expansion and resilience in aquaculture. Possible alternative feed resources include under-utilised organic wastes from agriculture and households and by-products from forestry. These resources usually have high nutrient content and may contain bioactive compounds (β-glucans, mannans, lignocellulose *etc*.) that elicit immunomodulatory responses, modulate gut microbiota and thus improve overall wellbeing in cultured fish. This thesis investigated the dietary potential of *Neurospora intermedia*, *Yarrowia lipolytica* and cello-oligosaccharides obtained from industrial by-products, municipal side-streams and forest by-products in diets for rainbow trout.

In a 30-day fish trial, *N. intermedia* was included in the diet by replacing 30% of the fishmeal-based control diet and diets containing fungi were processed with and without pre-conditioning (heat-treatment). The results showed high apparent digestibility coefficient of the *N. intermedia* diets and a gradual shift in overall gut microbiota, with increased abundance of *Lactococcus* from day 0 to 30. Preconditioning had no effect on digestibility or gut microbiota.

Pre-treated *Y. lipolytica* yeast biomass in whole (WY) or autolysed (AY) form was incorporated in rainbow trout diets at 2% or 5% level in a 45-day trial. The 5% WY diet resulted in elevated expression of immune-related genes of the complement pathway, membrane receptor pathway, cytokines and adaptive immune pathway. There was a small impact of dietary *Y. lipolytica* on faecal microbiota in rainbow trout.

The bioactivity of cello-oligosaccharides (COS) was examined by feeding rainbow trout diets containing 0-1.5% graded COS in a 45-day trial. Inclusion of 0.5-1.5% COS slightly increased lactic acid bacteria in faeces and marginally modulated gut immunity with respect to expression of complement and toll-like receptors. The COS diets also increased oxidative stress-reducing capacity in the gut and serum of the fish.

These results indicate, *N. intermedia*, *Y. lipolytica* and COS can be used successfully as potential functional feed or additive for rainbow trout.

Keywords: Filamentous fungi, Yeast, *Yarrowia lipolytica*, *Neurospora intermedia*, Cello-oligosaccharides, Microbiota, Mucosal immunity, Functional feed, biowaste

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Alternativa foderkällor: Effekter på tarmmikrobiota, immunitet och hälsa hos regnbåge

Abstract

Att byta från konventionellt använda fisk- och sojamjölsbaserade foder till hållbara och mer ekonomiska kostingredienser är avgörande för expansion och konkurrensskraft inom vattenbruket. Möjliga alternativa foderresurser inkluderar underutnyttjat organiskt avfall från jordbruk och hushåll samt biprodukter från skogsbruk och industri. Dessa har vanligtvis ett högt näringsinnehåll och kan också innehålla bioaktiva föreningar (β-glukaner, mannaner, lignocellulosa etc.), som genom att påverka immunförsvar och tarmmikrobiota kan förbättra det övergripande välbefinnandet hos odlad fisk. Denna avhandling undersökte möjligheten att använda alternativa kosttillskott för regnbågslax, d v s *Neurospora intermedia*, *Yarrowia lipolytica* och cello-oligosackarider, erhållna från industriella biprodukter, kommunala sidoströmmar respektive skogsbiprodukter.

N. intermedia inkluderades i kosten i en 30 dagar lång studie genom att ersätta 30 % av den fiskmjölsbaserade kontrolldieten och dieter som innehöll denna svamp bearbetades med och utan förkonditionering (värmebehandling). Resultaten visade en hög smältbarhet för dieter med *N. intermedia* och en gradvis förändring av den totala tarmmikrobiotan, med ökat andel av *Lactococcus* från dag 0 till 30. Förkonditionering hade ingen effekt på smältbarhet eller tarmmikrobiota.

Förbehandlad *Y. lipolytica*-jästbiomassa i hel (WY) eller autolyserad (AY) form inkorporerades i dieter på 2% eller 5% nivå i ett 45-dagars försök. 5% WY-dieten gav signifikant förhöjt uttryck av olika immunrelaterade gener medan de andra dieterna inte hade någon signifikant inverkan på genuttryck. En viss påverkan av dieter med *Y. lipolytica* på fekal mikrobiota hos regnbåge observerades.

Bioaktiviteten hos cello-oligosackarider (COS) undersöktes genom att utfodra regnbåge med dieter innehållande 0-1,5 % graderad COS i ett 45-dagars försök. Inkludering av 0,5-1,5% COS ökade andelen mjölksyrabakterier i feces och resulterade i viss effekt på tarmimmunitet med avseende på uttryck av komplement- och toll-liknande receptorer. COS-dieterna ökade också den oxidativa stressreducerande förmågan i fiskens tarm och serum.

Resultaten visar att kan *N. intermedia*, *Y. lipolytica* och COS användas framgångsrikt som funktionellt foder eller tillsats för regnbåge.

Nyckelord: Filamentösa svampar, Jäst, Yarrowia lipolytica, Neurospora intermedia, Cello-oligosackarider, Mikrobiota, Mukosal immunitet, Funktionellt foder, bioavfall

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Dedication

To my beloved parents

कर्मण्येवाधिकारस्ते मा फलेषु कदाचन।
मा कर्मफलहेतूर्भूर्माते सङ्गोऽस्त्वकर्मणि।।४७।।
"You are only entitled to the action, never to its fruits"
(Bhagavad Gita, Chapter 2, Verse 47)

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. Karimi, S., **Singh, A**., Langeland, M., Ferreira, J.A., Soofiani, N.M., Taherzadeh, M.J. & Vidakovic, A. Digestibility of the filamentous fungal biomass, *Neurospora intermedia*, in rainbow trout (*Oncorhynchus mykiss*). (Manuscript)

II. **Singh, A**., Karimi, S., Vidakovic, A., Dicksved, J., Langeland, M., Ferreira, J.A., Taherzadeh, M.J., Kiessling, A. & Lundh, T. (2021). Dietary filamentous fungi and duration of feeding modulates gut microbiota composition in rainbow trout (*Oncorhynchus mykiss*). *Frontiers in Marine Science*, 8, pp. 728569.

III. **Singh, A**., Vidakovic, A., Singh, A., Dicksved, J., Schnürer, A. & Lundh, T. *Yarrowia lipolytica* yeast as a dietary supplement for rainbow trout (*Oncorhynchus mykiss*): Modulation of gut microbiota, mucosal health and immunity. (Manuscript)

IV. **Singh, A**., Vidakovic, A., Hjertner, B., Krikigianni, E., Karnaouri, A., Christakopoulos, P., Rova, U., Dicksved, J., Baruah, K. & Lundh, T. Effects of dietary supplementation of lignocellulose-derived cello-oligosaccharides on growth performance, antioxidant capacity, immune response, and intestinal microbiota in rainbow trout (*Oncorhynchus mykiss*). (Manuscript)

The contribution of Aprajita Singh to the papers included in this thesis was as follows:

I. Planned part of the experiment, carried out feed preparation and formulation, conducted sampling, performed the statistical analyses and wrote the manuscript.

II. Planned part of the experiment, carried out feed preparation, and formulation, sampled gut materials, analysed gut microbiota, performed the statistical analyses and wrote the manuscript.

III. Planned the experiment, carried out feed preparation, feeding and collection of waste from fish, sampled gut materials, analysed microbiota, and gene expression, performed the statistical analyses and wrote the manuscript.

IV. Planned the experiment and partly carried out feed preparation, carried out feeding and collection of waste from fish, sampled gut materials, analysed microbiota, antioxidant parameters and gene expression, performed the statistical analyses and wrote the manuscript.

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Abbreviations

WG	Weight gain
FCR	Feed conversion ratio
SGR	Specific growth rate
HSI	Hepato-somatic index
VSI	Viscero-somatic index
CF	Condition factor
SOD	Superoxide dismutase
CAT	Catalase
GPx	Glutathione peroxidase
TLR	Toll-like receptor
CD4	Cluster differentiation 4
OTU	Operational taxonomic unit
ASV	Amplicon sequence variant
ADC	Apparent digestibility coefficient
PRR	Pattern recognition receptors
MAMP	Microbe associated molecular pattern
GALT	Gut associated lymphoid tissue
NMDS	Non- metric multidimensional scaling
PCoA	Principal coordinate analysis
PCA	Principal component analysis
LFC	Log fold change analysis
16S rRNA	16S ribosomal ribonucleic acid
LAB	Lactic acid bacteria

1. Introduction

1.1 Global and Swedish aquaculture – an overview

Global fisheries and aquaculture production reached a record level in 2020, of 214 million metric tonnes (mmt), of which aquaculture contributed 122.6 mmt, including 87.5 mmt of aquatic animals worth US$264.8 billion and 35.1 mmt of seaweed worth US$16.5 billion (FAO, 2022). Around 54.4 mmt were farmed in inland waters and 68.1 mmt came from marine and coastal aquaculture (Figure 1).

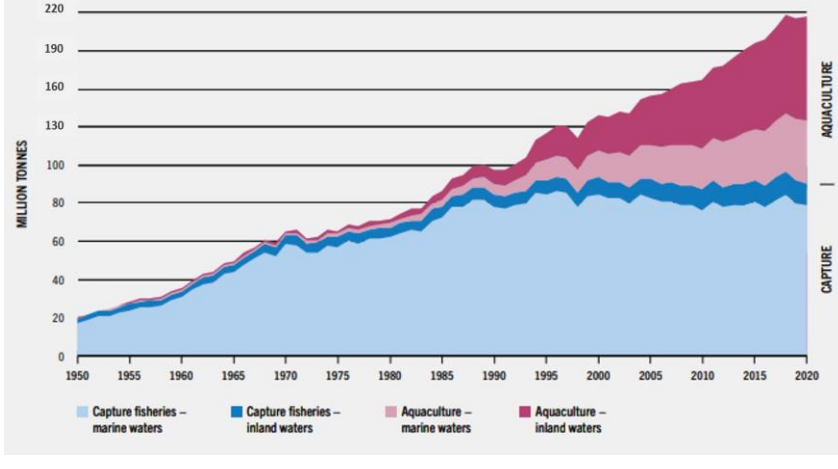


Figure 1. Global fisheries and aquaculture production (FAO, 2022).

Since 2000, global aquaculture production has increased by 5-7% annually, contributing ~50% to total fisheries production, while the overall aquaculture production in Europe has remained more or less stable and accounts for about 20% of fish and shellfish supply. Norway continues to be the leading aquaculture producer in Europe, with 58% of total monetary value, owing to its large-scale salmon production. However, in terms of volume the main aquaculture-producing European countries are Spain, France, Italy, Turkey, United Kingdom and Greece (FEAP, 2022).

Methods of aquaculture production in Europe are very diverse, consisting of sea cages, ponds, raceways and on-land recirculating aquaculture systems. In terms of farmed species in Europe, shellfish account for >45%, marine fish for >30% and freshwater fish for >20% of aquaculture production (European Commission, 2021). Despite the diversity of aquaculture production systems, the European countries are largely dependent on very few species, with mussels, salmon, seabream, rainbow trout, seabass, oysters and carp rank among the most significant (FEAP, 2022).

Sweden produces 0.2 mmt of fish and fishery products annually, making it a net importer of fish and fishery products (OECD, 2021). Swedish aquaculture contributes 27% of the domestic production and capture fisheries account for the remaining 73%. However, according to official statistics from the Swedish Agency for Agriculture, significant year-on-year growth of ~20% was seen during 2021, with total food fish production of 11,900 tonnes (Jordbruksverket, 2021). The dominant farmed species in Sweden is rainbow trout (*Oncorhynchus mykiss*), representing ~90% of total fish production, with Arctic char (*Salvelinus alpinus*) comprises the remaining 10%. In addition to fish, mussels contribute significantly to Sweden's annual aquaculture production, *e.g.*, 3500 tonnes of mussels were produced in 2021.

1.2 Aquafeed industry – challenges and opportunities

One of the many challenges facing the global food production industry is ensuring a steady supply of food and nutrition for the expanding global population while minimising waste. Fish is the most valuable and cheap source of dietary protein worldwide, with fisheries and aquaculture having the potential to ensure global food security (Ye *et al*., 2013). However, in order to feed the growing human population in future, more feed resources will be required to increase fish production. To date, fishmeal and fish oil have been the principal sources of proteins and lipids in aquaculture diets around the world. In particular, the dependency on fish-derived protein and lipid sources is much higher for the carnivorous fish species such as salmonids, as compared to omnivorous fish such as carp and tilapia (NRC, 2011). However, production of fishmeal and fish oil is strongly associated with sustainability issues related to marine capture fisheries and fluctuation in climate events, which in turn has led to elevated prices and lower availability (Bandara, 2018). Due to the sustainability issues, increased demand and elevated prices, the inclusion rate of fishmeal in aquafeeds has been decreasing over the years (Figure 2), and is being replaced with plant- and microbe-based protein sources (Agboola *et al*., 2021; Aas *et al*., 2022).

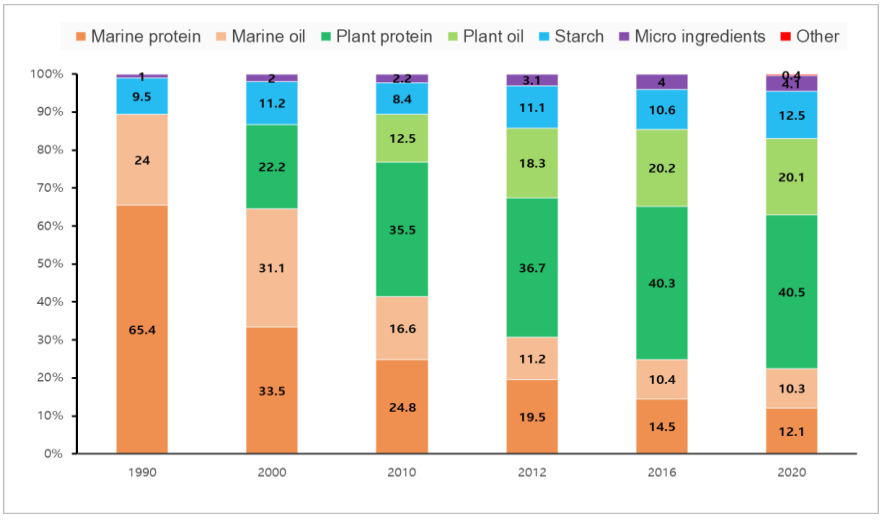


Figure 2. Changes in salmonid feed composition 1990-2020 (source: adapted from Aas *et al*., 2022).

However, conventional plant-based ingredients such as soy, mustard and rapeseed contain anti-nutritional factors that reduce growth in cultured fish (Krogdahl *et al*., 2010). Feed processing techniques can be applied to remove these inhibitory compounds from plant sources, such as soy protein concentrate, but this imposes additional costs for the production system (Hardy, 2010). The utilisation of conventional plant-based ingredients in aquafeed largely depends on their availability, after meeting demand in the terrestrial animal feed industry and human food chain (Francis *et al*., 2001; Bérot *et al*., 2005). Thus, for sustainable aquaculture production, there is an urgent need for alternative feed ingredients worldwide to make the industry more resilient and reduce dependency on conventional protein sources (Bandara, 2018).

1.3 Sustainable aquafeed – towards circular bioeconomy

Unconventional feed ingredients, such as forest by-products, insect by-products, terrestrial animal by-products, microbial ingredients and genetically modified ingredients, are being widely tested in efforts to meet the global demand for alternative sources of fish feed (Bandara, 2018). These alternative feed ingredients usually have a good amino acid profile suitable for the requirements in fish. Moreover, since they are by-products or waste materials, their utilization will ultimately lower the ecological impact and address the sustainability and environmental issues at the same time (Kardung *et al*., 2021).

An economic model based on production of biological resources from domestic, industrial or forest waste and appropriate use of these in different applications in the food, agriculture and allied sectors is termed a circular bio-economy (Mohan *et al*., 2016). Reusing or recycling waste as part of a circular bio-economy is a viable strategy to reduce reliance on imported natural resources, protect the environment and promote public health (Wesseler & von Braun, 2017). In the past decade, there has been a growing interest on applying the bio-economy model in the aquafeed industry, to use waste or by-products from forest and sewage streams as renewable supplementary feed resources (Bandara, 2018).

According to Statistics Sweden (2020), the volume of annual non-hazardous household waste generated in Sweden in 2020 was estimated as 7.4 mmt. In addition, forest cover comprising ~87 million trees, occupying to ~57% of the total land area in Sweden, contributes about 1.04 mmt waste annually. In combination, these municipal and forest waste resources make Sweden a suitable destination for a circular bio-economy. These wastes contain a variety of complex organic substances such as soluble carbohydrates, proteins, fats and fatty acids, and can therefore act as ideal substrates for fermentation and growth of microorganisms including, bacteria, yeast and filamentous fungi (Aggelopoulos *et al*., 2014; Gervasi *et al*., 2018; Kumar *et al*., 2021). The aquafeed industry is taking interest in utilising these microorganisms as feed resources for nutrients and value-added products (organic acids such as citric and lactic acid, enzymes, amino acids, metabolites), to play a crucial role in the bio-economy.

1.4 Alternative aquafeed sources

Potential alternative feed ingredients include filamentous fungi, yeasts, single-cell protein, insect protein and non-digestible oligosaccharides. They commonly have a very rich nutrient profile and also contain one or more bioactive compounds, such as β-glucan, mannan, chitin, lignocellulose or fructose, which can act as potential immunostimulants and modulate gut microbiota in fish (Free, 2013; Karimi *et al*., 2018; Zhong *et al*., 2020). In this thesis, three such ingredients (*Neurospora intermedia*, *Yarrowia lipolytica* and cello-oligosaccharides), produced from different sources (Figure 3 & 4), were tested as alternative feed ingredients in the diet of rainbow trout.

1.4.1 Filamentous fungi: *Neurospora intermedia*

Neurospora intermedia is a food-grade filamentous ascomycetous fungus first isolated from traditional fermented food in Indonesia. It can be grown on thin stillage, dairy waste or lignocellulosic biomass (Ferreira *et al*., 2014; Nair, 2017; Mahboubi *et al*., 2017). The United States Food and Drug Administration (USFDA) has deemed *N. intermedia* as GRAS (generally regarded as safe) for use in both animal feed and human food (Ferreira *et al*., 2013). With the aid of sophisticated enzyme machinery, this

fungus can survive on a variety of substrates by breaking down complex carbohydrates, lipids and proteins into simpler sugars, fatty acids and amino acids, respectively (Ferreira *et al*., 2013). *N. intermedia* has a high protein content, 50-60% (wet weight basis), with a good amino acid profile similar to that of fishmeal, thus, making it a promising alternative protein source for aquafeed (Archer *et al*., 2008; Ferreira *et al*., 2014; Karimi *et al*., 2018; Karimi *et al*., 2019). In addition to its nutritional value, microbial biomass from *N. intermedia* has also been investigated for its potential to produce bioactive compounds that can improve fish health and growth. According to Nimrichter *et al*. (2005), the cell wall of fungi are complex structures made up of 80-90% polysaccharides. The cell wall contains several bioactive compounds, such as glucan (30-80%), chitin and chitosan (1-15%), mannan and/or galactomannan, and glycoproteins (Free, 2013; Karimi *et al*., 2018).

1.4.2 Dietary yeast: *Yarrowia lipolytica*

Yarrowia lipolytica is a dimorphic ascomycetous yeast ubiquitously found in waste waters, soil sediments, marine ecosystems and other environments with a high content of fats or hydrocarbons (Barth & Gaillardin, 1997; Beopoulos *et al*., 2009; Zinjarde *et al.*, 2014). There are several useful biotechnological and environmental applications for its whole biomass and various derivatives (Bankar *et al*., 2009; Liu *et al*., 2015). As it is a rich source of essential fatty acids, *Y. lipolytica* is used to make polyunsaturated fatty acids (PUFAs), including docosahexaenoic acid (DHA; 22:6n-3) and eicosapentaenoic acid (EPA; 20:5n-3) (Carsanba *et al*., 2018). It is also used for production of diverse groups of enzymes such as lipases, esterases, proteases and phosphatases and other important metabolites (polyamines, citric acid), both by *de novo* synthesis and by uptake from exogenous sources (Ong *et al*., 2019; Liu *et al*., 2019). The high protein content (30-70%) and high levels of essential amino acids (lysine, phenylalanine, valine, tryptophan and isoleucine), vitamins (B1, B2, B6, pantothenic acid, niacin, folic acid and biotin), minerals and other immunostimulants such as β-glucan (50-60%), chitin (1-2%) and mannoproteins have led to interest in *Y. lipolytica* as a potential functional feed (Morgunov *et al*., 2018; Gálvez-López *et al*., 2019; de Marco Castro *et al*., 2021). GRAS recognition of its biomass and other derived compounds by European and American agencies has escalated its use in applications in

the feed and food industry (Turck *et al*., 2019). In fish species, dietary supplementation with *Y. lipolytica* is reported to improve the essential fatty acid profile, digestibility and bioavailability of EPA and DHA in Atlantic salmon (*Salmo salar*) (Hatlen *et al*., 2012; Berge *et al.,* 2013). Immunomodulatory properties have also been reported in Pacific red snapper (*Lutjanus peru*) (Alamillo *et al*., 2017) and Nile tilapia (*Oreochromis niloticus*) (Neuls *et al*., 2021). In zebrafish (*Danio rerio*) and Pacific red snapper, *Y. lipolytica* has been found to have probiotic and immune-protective effects against the pathogenic bacterial species *Aeromonas* and *Vibrio* (Caruffo *et al*., 2016; Reyes-Becerril *et al*., 2021).

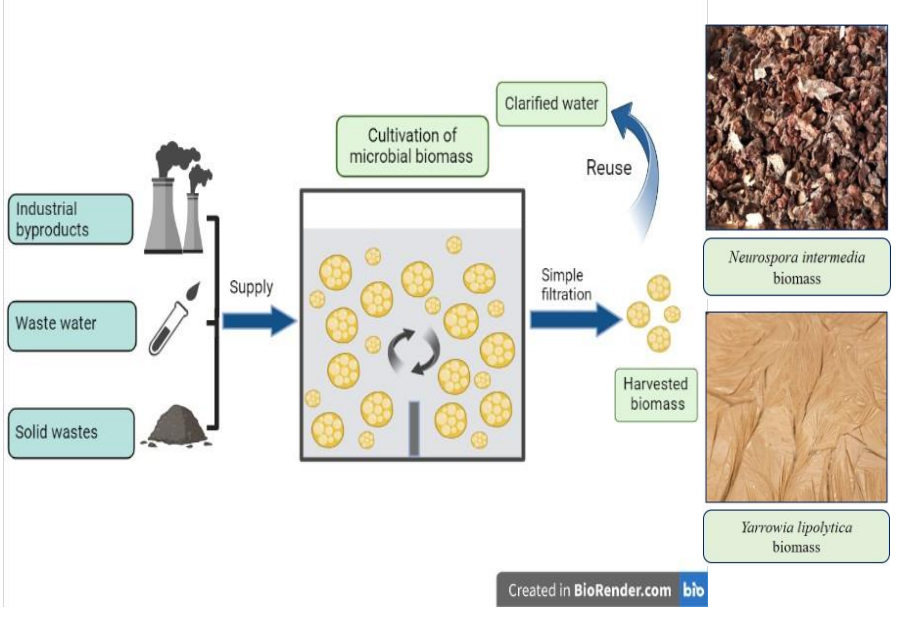


Figure 3. Sustainable cultivation of microbial biomass from waste streams.

1.4.1 Prebiotic: Cello-oligosaccharides (COS)

Cello-oligosaccharides are linear, non-digestible oligosaccharides that can be produced by controlled enzymatic hydrolysis of cellulose (Figure 4). They are made up of 3-10 short-chain β-(1,4) glucopyranose units (Karnaouri *et al*., 2019; Barbosa *et al*., 2020). Cello-oligosaccharides and cellobiose (a dissacharide) are produced from lignocellulosic biomass of currently under-utilised agricultural by-products and forest residues by enzymatic degradation (Karnaouri *et al*., 2019). Lignocellulose is an abundant, natural, renewable and cheap resource. It is a heterogenous complex of the carbohydrate polymers cellulose and hemicellulose and the aromatic lignin (McKendry, 2002). Cello-oligosaccharides (COS) have great potential as prebiotic in higher vertebrates, such as cattle, pigs and humans, where their beneficial effects on host digestion and intestinal ecology have been reported (Satouchi *et al*., 1996; Otsuka *et al*., 2004; Song *et al*., 2013; Uyeno *et al*., 2013; Zhong *et al*., 2020). Their potential health benefits, as manifested in humans and farm animals, derive from their ability to promote growth of lactic acid bacteria (LAB) *in vitro* and *in vivo* (Kontula *et al*., 1998; Karnaouri *et al*., 2019; Canigiano *et al*., 2020). LAB are involved in production of short-chain fatty acids such as lactate, butyrate and bacteriocins and prevent adherence and colonisation of pathogenic bacteria (Ringo *et al*., 2018; Li *et al*., 2019). Hence, COS can be a viable, innovative and environmentally friendly fish feed additive.

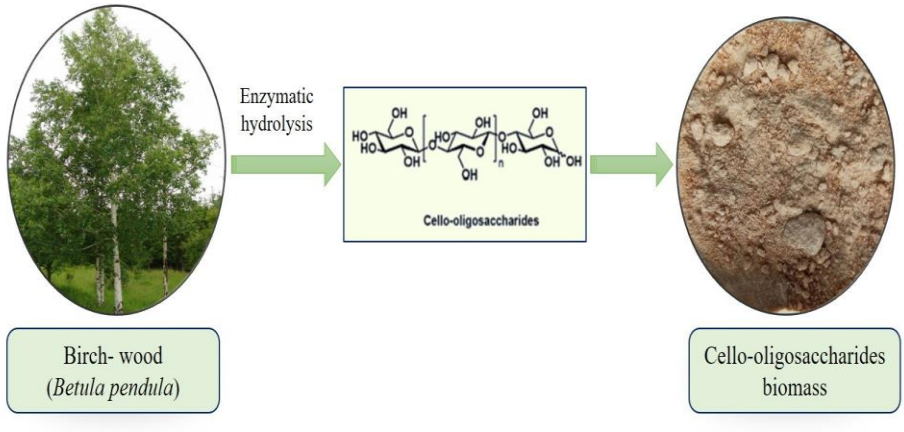


Figure 4. Cello-oligosaccharides from enzymatic hydrolysis of birchwood residues.

1.5 Alternative aquafeed and fish health

A crucial first step in successful formulation of functional feeds is evaluation of the potential feed ingredients in terms of their impact on digestibility, growth and gut health. The gastrointestinal tract, which first interacts with the feed, serves as an absorptive surface for the nutrients or molecules carried in feed. These nutrients interact with gut microbiota and modulate the mucosal immunity directly by activating the gut's immune receptors or by producing microbial metabolites.

1.5.1 Digestibility and growth

Digestibility of a nutrient or energy is a measure of how much of that nutrient or energy is absorbed by the animal. In modern diets, digestible energy and digestible nutrients are more important than gross values of these (Cho & Kaushik, 1990). Determination of digestibility is the first step in evaluating the potential of an ingredient for use in the diet of an aquaculture species. Measured digestibility depends on the type of diet, downstream processing of ingredients, feed processing methods such as extrusion or cold pelleting, feeding strategy and faeces collection method (Glencross *et al*., 2007). For example, microbial biomass, which has a thick and rigid cell wall, prevents enzyme access to the cellular components and release of nutrients from the cell, thus lowers the digestibility of the microbial ingredient. Downstream processing of yeast biomass can increase the digestibility, as shown in a study by Rumsey *et al*. (1990), where they reported intact cell biomass of *Saccharomyces cerevisiae* had poor protein and amino acid digestibility in rainbow trout compared with disrupted cell biomass. In another study, disruption of intact cell walls of *S. cerevisiae* showed a positive effect on yeast protein digestibility in Arctic char (Langeland *et al*. 2016).

Growth, a measure of net nutrient deposition, is the most common response to reflect changes in nutrient content in the diet. Type of diet, temperature, downstream processing of feed, inclusion level, sex, age, rearing conditions and feeding habit can influence fish growth (Viadero, 2005; Soderberg, 2017). Previous studies have shown that a high level of yeast replacing fishmeal can have a negative effect on digestibility and growth, but upon processing of the yeast improves the growth performance

(Øverland & Skrede, 2017). A study by Ozório *et al.* (2010) demonstrated improved growth performance when a moderate level (250 g kg^{-1}) of *S. cerevisiae* was included in the diet of rainbow trout, while high inclusion levels (500 g kg^{-1} and 750 g kg^{-1}) reduced feed intake and growth.

1.5.2 Gut microbiota

The fish gut microbiota plays an important role in gastro-intestinal (GI) tract development, digestive function, mucosal tolerance, stimulation of the host immune response and protection against infections (Gómez & Balcázar, 2008; Ray *et al*., 2012; Piazzon *et al*., 2017; Li *et al*., 2018; Wang *et al*., 2018). However, there is a weaker understanding of gut microbiota in fish than in humans and other higher animals. In general, Proteobacteria is found to be the dominant phylum in most fish microbiota, with Fusobacteria constituting another abundant taxon in other cases, although variation exists between different fish species (Kim *et al*., 2021). The gut-associated microbes have potential beneficial or harmful effects on the host. An imbalanced microbiota can negatively affect fish nutrition and growth and lead to alteration of gut immune functions, contributing to the development of disease. Therefore, a better understanding of gut-microbe interactions and gut microbial diversity in fish would be highly relevant for aquafeed processing.

Gut microbiota has been shown to regulate many key aspects of the host's body functions, including feeding behaviour, energy balance, nutrient metabolism and immune response (Wang *et al*., 2018; Butt & Volkoff, 2019). Further, gut microbiota composition exhibits a close relationship with the activity of the intestinal digestive enzymes of fish, such as cellulase, amylase, trypsin and lipase (Liu *et al*., 2021). Fish gut microbiota also shows chitin-, cellulose-, and collagen-degrading ability (Kar & Ghosh, 2008). Likewise, the gut microbiota has been shown to promote energy absorption, regulate the expression of genes related to energy and lipid metabolism, and aids in digestion and absorption of fat in fish (Semova *et al*., 2012). Complex and integrated interactions between the epithelium, mucosal immune components and the gut microbes play a critical role in development and maturation of gut-associated lymphoid tissues (GALT), which in turn mediate a variety of host immune functions (Rhee *et al*., 2004).

Interplay of a variety of factors influences gut microbiome composition and diversity, and its function and metabolic activity, thus affecting feeding, growth, energy storage and health of the fish. The structure of fish gut microbiota is affected by biotic (host gene makeup, developmental stage, feeding habits, age) and abiotic (water condition, diets) factors (Hovda *et al*., 2012; Ingerslev *et al.*, 2014; Li *et al*., 2016; Ringø *et al*., 2018; Yan *et al*., 2016; Sun *et al*., 2020). Rimoldi *et al*. (2018) found that plant-derived proteins favoured a higher Firmicutes: Proteobacteria ratio in the intestinal microbiota of rainbow trout compared with animal proteins. Plant-derived dietary proteins have also been linked to an increase in relative abundance of *Lactobacillales*, *Bacillales* and *Pseudomonadales*, while animal-derived proteins have been shown to promote *Bacteroidales*, *Clostridiales*, *Vibrionales*, *Fusobacteriales* and *Alteromonadales* in the gut of rainbow trout (Michl *et al*., 2017). Further, Gajardo *et al*. (2017) showed adverse effects of high levels of soy protein in the diet on gut microbial composition in Atlantic salmon, leading to inflammation of the distal intestine.

Based on these studies, inclusion of alternative feed sources appears to influence the host's gut microbiota. Therefore, this thesis examined modulation of gut microbiota in response to *N. intermedia*, *Y. lipolytica* and cello-oligosaccharides in the diet of rainbow trout.

1.5.3 Immune gene modulation

The fish gut plays a crucial role in osmoregulation, and nutrient absorption, and acts as the body's main defence against the external environment (Xu *et al*., 2021). The microbiota, the mucus layer, the physical barrier made up of enterocytes and the underlying GALT form the main components of gut mucosal immunity (Nayak, 2010). The mucus secreted by enterocytes contains lysozymes, antimicrobial peptides and immunoglobulins, which are considered as the first line of defence against pathogen attack (Uribe *et al*., 2011). Fish GALT, comprising of lymphocytes, eosinophilic granular cells, granulocytes and plasma cells, are involved in innate and acquired immunity (Rombout *et al*., 2011). Growth and maturation of GALT are significantly influenced by gut microbes (Rhee *et al*., 2004). For instance, probiotic supplementation has been shown to increase the numbers of gut T-lymphocytes and granulocytes and modulate immune genes in fish at early developmental stages (Picchietti *et al*., 2009).

Pro-inflammatory cytokines, such as tumour necrosis factor-α (*tnf-α*), and interleukin-1β (*il-1β*), start the inflammatory process through T-lymphocyte pathways in salmonids (Uribe *et al*., 2011). These cytokines induce other innate immune cells such as neutrophils and macrophages, which are capable of secreting antimicrobial compounds and phagocytising microorganisms. Anti-inflammatory cytokines, such as transforming growth factor-β (*tgf-β*) and *il-10*, are induced in order to balance the innate immune response and avoid an overreaction (Zhang *et al*., 2009). Previous studies have demonstrated that low inclusions of live *S. cerevisiae* yeast in the diet reduce intestinal inflammation (increased microvilli length and density) in Nile tilapia, and decrease relative expression of pro- and anti-inflammatory cytokines (*tnf-α* and *tgf-β*) (Ran *et al*., 2015). Conversely, high inclusions of inactivated yeast (40-60% replacement of fishmeal) are reported to impair gut barrier function and cause oedema of microvilli in Arctic char and rainbow trout (Vidakovic *et al*., 2016).

Beside inclusion levels, alternative feed sources possessing certain bioactive compounds, such as β-glucan and chitin from yeast and fungi and oligosaccharides (cellulose, fructose, mannose) from forest residues, are known to influence the gut mucosal immunity *via* two pathways, by acting as an immunostimulant or by augmenting gut microbiota, which in turn acts as an antigen for activation of immune response. Both these pathways involve presentation and binding of the pathogen-associated molecular pattern (PAMP) such as β-glucan (from yeast) or lipopolysaccharides (from augmented bacteria in the gut) to the pattern recognition receptors (PRRs), followed by downstream signalling of cytokines and activation of gut mucosal immunity (Hassan, 2011; Shurson, 2018). Several PRRs, including dectin-1, toll-like receptors, c-type lectin receptor and complement receptor type-3 (cr3), are involved in the corresponding pathways of fish mucosal immunity (Boshra *et al*., 2006; Dalmo & Bøgwald, 2008; Rebl *et al*., 2010; Ji *et al*., 2020).

Studies on the expression of genes of different receptor-based immune pathways and the signalling cytokines could thus help in understanding the role of alternative feed sources in terms of gut immunomodulation in fish. For this reason, the expression of immune genes in the intestine of rainbow trout fed diets containing *N. intermedia*, *Y. lipolytica* and cello-oligosaccharide was studied in this thesis.

2. Aims of the thesis

Considering the need for alternative strategies in fish health management, together with the great potential effect of novel feed resources obtained from municipal or industrial side-streams or forest by-products, the main aim of this thesis was to test the use of alternative feed sources as a supplement in fish diets. The effects of three alternative feed sources, *Neurospora intermedia*, *Yarrowia lipolytica* and cello-oligosaccharides, obtained from industrial by-products, municipal side-streams and forest by-products, respectively, on gut health and overall wellbeing of rainbow were investigated.

Specific objectives in feeding experiments (Papers I-IV) conducted to evaluate the efficiency of these feed sources in the diet of rainbow trout were as follows:

- To investigate the potential of *Neurospora intermedia* as a protein source for rainbow trout, based on digestibility and gut microbiota analysis (Papers I and II).
- To determine the modulatory effect of inclusion of *Yarrowia lipolytica*, as a functional additive, on gut microbial composition and mucosal immunity in rainbow trout (Paper III).
- To investigate the potential of cello-oligosaccharides, as a functional additive, on gut microbiota, mucosal immunity and stress parameters in rainbow trout (Paper IV).

3. Materials and methods

3.1 Fish rearing and maintenance

The experiments reported in Papers I-IV were carried out in the Aquatic Facility at the Centre of Veterinary Medicine and Animal Science, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden. Fish were procured from a commercial fish farm, Vilstena Fiskodling AB, Fjärdhundra, Sweden, and raised in 500-1000 L holding tanks. In the experiments, a total of 300, 270 and 225 juvenile rainbow trout were distributed between 200-L experimental tanks, with n = 20, 18 and 15 fish/tank in Papers I and II, Paper III and Paper IV, respectively. The tanks were fitted with waste belt collectors (Hølland teknologi, Sandnes, Norway) and partial water recirculation systems, where tank water was replaced by fresh municipal water at a rate of 3 L min^{-1}. Rearing conditions were maintained at 11±1 °C with a 12 h dark/12 h light photoperiod (from 08:00 to 20:00 h), and dissolved oxygen concentration ranged between 9 and 10 mg L^{-1} (measured by HQ40D Portable Multi Meter, Hach, Loveland, CO, USA) during the whole experiment. Before the start of all experiments, fish were acclimatised for 14 days in the experimental tanks, during which they were fed a commercial diet (INICIO 917, Biomar, Denmark), twice per day at 2% of body weight.

3.2 Experimental design

The studies reported in Papers I-IV were all performed with rainbow trout fed diets based on the different test ingredients: *Neurospora intermedia* (Papers I and II), *Yarrowia lipolytica* (Paper III) and cello-oligosaccharides

(COS) (Paper IV). Throughout the studies, sampling of blood serum, gut contents, and distal gut tissue was performed for analyses of gut microbiota, digestibility, antioxidant activity and/or gene expression (see Table 1 for more information).

Table 1. Details of the experiments carried out in Papers I- IV in this thesis

	Papers I, II	Paper III	Paper IV
Fish species	Rainbow trout	Rainbow trout	Rainbow trout
Initial body weight	127.8 ± 19.8 g	30.0 ± 10.0 g	30.5 ± 10.2 g
Period	30 days;	45 days;	45 days;
	Multiple sampling	Final sampling	Final sampling
Water temperature	11±1 °C	12±1 °C	11±1 °C
Number of diets	3	5	5
Replicates, tanks	5, 15	3, 15	3, 15
Test ingredient	*Neurospora intermedia*	*Yarrowia lipolytica*	Cello-oligosaccharide
Feed production	Cold-pelleting	Heat-extrusion	Cold-pelleting
Material sampled	Faeces, Distal gut	Distal gut content & tissue	Distal gut content & tissue, serum
Analyses	Growth indices, gut microbiota, digestibility	Growth and body indices, gut microbiota & gene expression	Growth indices, antioxidants, gut microbiota & gene expression

3.3 Test ingredients

3.3.1 *Neurospora intermedia*

Fungal biomass of *N. intermedia* CBS 131.92 (Centraalbureau voor Schimmelcultures, The Netherlands) was produced under semi-continuous cultivation conditions in a 26 L capacity bubble column bioreactor (airlift bioreactor converted to bubble column bioreactor by removing the internal loop tube; Bioengineering, Switzerland) at the Swedish Centre for Resource Recovery, University of Borås. The fungus was cultivated on complex medium containing 30 g L^{-1} glucose and 5 g L^{-1} yeast extract as the major carbon and nitrogen source, respectively. Trace elements (in the form of $(NH_4)_2SO_4$, KH_2PO_4, $CaCl_2.2H_2O$ and $MgSO_4$ $x7H_2O$ in concentrations of 7.5, 3.5, 1.0 and 0.75 g/L, respectively) were added to the cultivation medium to support filamentous fungi growth. Cultivation was carried out at 35 °C and 1 volume of air per volume of medium per minute (vvm). Cultivation condition parameters and sterilisation method were according to Ferreira *et al*. (2015). To obtain biomass, 75% of the working volume of the reactor (15 L) was harvested twice per day, at 11.00 and 23.00 h. Fresh sterilised cultivation medium was added to top up the cultivation broth after harvesting. Harvested broth, containing post-cultivation medium and biomass, was transferred to a cold room and stored at 4 °C. After termination of cultivation, biomass was quickly separated from the culture medium using a sieve, washed with distilled water and dried overnight in a hot air oven at 70 °C.

3.3.2 *Yarrowia lipolytica*

The yeast strain used in Paper III was *Y. lipolytica* CBS 7504, originally isolated from sewage at a wastewater treatment plant in Uppsala, Sweden. The *Y. lipolytica* CBS 7504 strain was inoculated in YPD broth medium (maintained at pH 6.2), containing 27 g L^{-1} sodium acetate, 20 g L^{-1} sodium DL-lactate, 2.6 g L^{-1} sodium propionate, 1.7 g L^{-1} yeast nitrogen base (without amino acids and ammonium sulphate), and 5 g L^{-1} ammonium sulphate, and incubated for 24 h at 30 °C in an orbital shaker. Precultured yeast was harvested from the medium after reaching the exponential phase (24 h post-inoculation), by centrifugation at 1000 × *g* for 10 min at 4 °C, and washed twice with saline (0.9% NaCl). The yeast pellet was collected by decanting the saline medium, after which it was inoculated in 1.5 L YPD

broth medium containing 20 g L^{-1} acetic acid, 16 g L^{-1} DL-lactic acid, 2 g L^{-1} propionic acid, 1.7 g L^{-1} yeast nitrogen base and 5 g L^{-1} ammonium sulphate, in a Dolly bioreactor (6 L working volume; Belach Bioteknik AB, Huddinge, Sweden) for continuous cultivation. The continuous culture was maintained at pH 3.5 using the pH-stat method, by connecting the feed medium with acid titrant (pH set at 6.2 with a 0.10 deadband) fed through a 0.2 μm sterile filter (Sartolab P20 plus, Sartorius Stedim). The culture was aerated using compressed air, with a pO_2 set-point at 20%, maintained by stirring speed. To compensate for acidification of the medium due to ammonium utilisation, aliquots of 5 M NaOH were added manually throughout the cultivation to maintain pH. A maximum working volume of 5 L was maintained by pumping out liquid using a tube positioned at the corresponding height inside the bioreactor. The yeast biomass was harvested when the culture reached the target optical density of 1.3, by centrifugation at 1000 × g for 30 min at 4 °C. After decanting the supernatant, the yeast biomass was washed twice with deionised water by centrifugation at same settings. The harvested yeast cell biomass (1.4 Kg wet weight, corresponding to 278 g dry weight) was divided into two equal parts. One part was air-dried at room temperature (15 °C) for whole yeast and the other part was subjected to autolysis before drying. For the autolysis process, yeast biomass was heated to 50 °C for 16 h under continuous stirring (200 rpm). After autolysis, the disrupted cells were freeze-dried and stored at -20 °C.

3.3.3 Cello-oligosaccharides

Lignocellulose-derived cello-oligosaccharide (COS) was prepared by enzymatic hydrolysis of organosolv-pre-treated birchwood (*Betula pendula*) using the commercially available enzyme mixture Celluclast® (Sigma-Aldrich, USA), according to a previously described protocol (Karnaouri *et al*., 2019). The COS powder contained a mixture of cello-oligosaccharides and cellobiose disaccharide (13.5% on dry weight basis) and a negligible level of glucose (<0.1%).

3.4 Diets and feeding

Feed preparation was performed in the Feed Technology Laboratory at SLU, Uppsala, Sweden. In Papers I, II and IV, feeds were prepared by cold pelleting using a 3.5 mm die (Nima Maskinteknik AB, Örebro, Sweden). In

Paper III, feed was prepared by extrusion using a twin-screw extruder Brabender KETSE 20/40 (Brabender GmbH & Co. KG, Duisburg, Germany) equipped with five heating zones and a 2 mm die head (for details of cold pelleting, extrusion conditions and feed composition for different diets, see Material & Methods section in Papers I-IV).

In Papers I and II, three experimental diets were prepared: a reference diet (RD) and two test diets (non-preconditioned (NPD) and preconditioned (PD) (note that these same diets are denoted CD, NI and PNI, respectively, in Paper I). RD was prepared with fishmeal as the major protein source, while PD and NPD were prepared by replacing 30% of RD diet with *N. intermedia* (Cho, 1979). Preconditioning of PD was performed by heat-processing in a convection oven (Electrolux Professional, FCE061) at 105 °C for 5 min prior to feeding.

In Paper III, a control diet containing no yeast and five iso-nitrogenous and iso-energetic experimental diets with 2% and 5% (w/w) inclusion of whole yeast (WY) or autolysed *Y. lipolytica* yeast (AY) were prepared. All diets were formulated to slightly exceed the minimum nutritional requirements of rainbow trout (NRC, 2011).

In Paper IV, five experimental diets were prepared: a control diet without inclusion of any prebiotic compound; a positive control diet with 0.5% (w/w) inclusion of fructo-oligosaccharides (FOS) (Sigma-Aldrich, USA); and three diets with different inclusion levels (w/w) of COS (COS 0.1%, COS 0.5%, COS 1.5%).

Fish were fed twice a day throughout the 30-day feeding trials in Papers I & II (at the rate of 2% body weight; bw) and 45-day feeding trials in Paper III (1.5% of bw) & IV (2% of bw), using automatic belt feeders (Hølland teknologi, Sandnes, Norway). Feed ration was adjusted based on estimated thermal growth coefficient (Cho, 1992).

3.5 Proximate composition analysis

Following preparation of the different experimental diets and test ingredients in Papers I-IV, samples of each feed were freeze-dried, milled and stored at -20 °C for proximate composition analysis. To determine dry matter content of both feed and feed waste, samples were dried in a hot-air

oven for 16 h at 103 °C and then cooled in a desiccator before weighing. All experimental feeds were analysed for crude protein content (nitrogen, N × 6.25) by the Kjeldahl method (Nordic Committee on Food, 1976), using a 2020 Digester (with Cu as catalyst) and 2400 Kjeltec Analyser unit (FOSS Analytical A/S, Hilleröd, Denmark). Crude lipid content was analysed according to the Official Journal of the European Communities (1984), using a Soxhlet extraction unit (1047 Hydrolysing Unit, Soxtec System HT 1043, FOSS Analytical A/S). Neutral detergent fibre (NDF) was measured based on the method described by Chai and Udén (1998), using a 100% neutral detergent solution, with amylase and sulphite used for reduction of starch and protein. Gross energy content was determined in an isoperibol bomb calorimeter (Parr 6300, Parr Instrument Company, Moline, IL, USA). Dry matter content and ash content were determined according to standard methods (AOAC, 1995). For detailed proximate composition data of diets and test ingredients, see Paper I-IV.

3.6 Sampling

Initial and final body weight of fish from each treatment were measured at the start and end of the feeding trial for determination of growth parameters. Before handling, fish were anaesthised or euthanised using tricaine methane sulfonate (MS-222, Western Chemical Inc., Ferdale, WA, USA). For the digestibility study in Paper I, faeces were collected and stored at -20 °C until analysis. Proximate analysis of faeces was performed for 10-, 20- and 30-day samples. For microbiota analyses, in Paper II five fish per treatment were sampled at day 0, 10, 20 and 30 of feeding, while in Papers III and IV nine fish per treatment were sampled at day 46.

For gut microbiota analysis in Papers I-IV, fish were aseptically dissected from the ventral side after swabbing with ethanol (70% solution). The hindgut was dissected from the ileocaecal valve to 0.5 cm above the anus, and digesta samples and/or mucosal scrapings were taken. The samples were snap-frozen in liquid nitrogen and stored at -80 °C until DNA extraction.

In Paper IV, blood was collected from the caudal vein of three fish for each treatment using a non-heparinised syringe, kept at 22 °C for 30 min for clotting, followed by centrifugation at 2000 × g at 4 °C for 15 min. The

serum samples were collected and stored at -80 °C until use for antioxidant enzyme activity assay. In Paper III, liver and viscera (whole GI tract, with stomach and pyloric caeca) were dissected out from the fish for determination of hepato-somatic index and viscero-somatic index, respectively.

In Papers III and IV, the distal segment of six intestinal samples per treatment was collected and stored in RNAprotect Tissue Reagent (Qiagen, Germany) for 24 h at 4 °C and later stored at -20 °C until RNA extraction for gene expression analysis.

3.7 Analyses

3.7.1 Growth parameters and biometric indices (Papers I-IV)

Four growth parameters, survival percentage (percentage weight gain (WG), feed conversion rate (FCR), specific growth rate (SGR) and) and three body indices (hepato-somatic index (HSI), viscero-somatic index (VSI) and condition factor (CF) were calculated using the following equations:

$$WG\ (\%) = [\text{Final weight (g)} - \text{Initial weight (g)}]/\text{Initial weight (g)} \times 100$$

$$FCR = \text{Feed intake (g)}/\text{Weight gain (g)}$$

$$SGR = [\ln(\text{Final weight}) - \ln(\text{Initial weight})]/\text{Time (days)} \times 100$$

$$\text{Survival}\ (\%) = [\text{Number of fish at end}/\text{Number of fish at start}] \times 100$$

$$HSI = \text{Weight of liver (g)}/\text{Weight of fish (g)} \times 100$$

$$VSI = \text{Weight of viscera (g)}/\text{Weight of fish (g)} \times 100$$

$$CF = \text{Weight of fish (g)}/[\text{Total length of fish (cm)}]^3 \times 100$$

3.7.2 Digestibility (Paper I)

Dietary apparent digestibility coefficient (ADC) was calculated as (Cho *et. al*., 1979):

$$ADC_{diet} = 1 - (F/D \times D_i/F_i)$$

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:

$$ADC_{ingred} = ADC_{test} + [(ADC_{test} - ADC_{con}) \times (0.7 \times D_{con} / 0.3 \times D_{ingred})]$$

where D_{con} is percentage nutrient content (or kJ g^{-1} gross energy) in the control diet (as-is) and D_{ingred} is percentage nutrient content (or kJ g^{-1} gross energy) in the fungal biomass (as-is basis).

3.7.3 Serum antioxidant analysis (Paper IV)

Activity of the specific antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) in the experimental serum samples was measured by colorimetric assay, using the respective Antioxidant Assay Kit (Elabscience Biotechnology Inc., Houston, TX, USA) according to the manufacturer's protocol (Catalogue No: E-BC-K019-M, E-BC-K031-M, E-BC-K096-M, respectively). All biochemical analyses were performed in duplicate.

3.7.4 DNA extraction (Papers II-IV)

Faeces samples (10-100 mg) were transferred to sterile cryotubes containing 1 mL of InhibitEX buffer (Qiagen, Germany) and 0.5 g silica beads (0.1 mm diameter). The samples were homogenised by two runs in a Precellys Evolution homogenizer (Bertin Technologies, France) at 6000 rpm for 1 min, with a 5 min interval on ice. DNA was isolated from the homogenised samples using a QIAamp Fast DNA Stool Mini Kit (Qiagen, Germany) according to the manufacturer's protocol.

3.7.5 Gut microbiota analysis (Papers II-IV)

Extracted DNA was PCR-amplified with barcodes and a DNA library was prepared and sequenced using Novoseq 6000 at Novogene (China or United Kingdom). The PCR reactions consisted of extracted DNA, Phusion® High-Fidelity Master Mix (Thermo Fisher Scientific Inc., Waltham, MA, USA) and 341F or 515F and 805R Illumina primers that targeted the V3 or V4 region of 16S rRNA gene. Amplicons were PCR-amplified again in order to individually barcode each sample. Samples were quantified using a Qubit 3.0 Fluorometer (Invitrogen, Thermo Fisher Scientific).

Alpha-diversity of bacterial operational taxonomic unit (OTUs) was determined using the Shannon and Chao-1 richness indices with Paleontological Statistics (PAST) software (Hammer *et al.*, 2001). Beta-diversity of bacterial OTUs was determined using principal coordinate analysis (PCoA) in Paper II and principal component analysis (PCA) with PAST software in Paper IV. In Paper III, differential abundance analysis was performed with the package *DESeq2* (v1.34.0) and alpha-diversity of amplicon sequence variants (ASVs) was determined using the estimate richness function in *phyloseq* with the Observed, Shannon and Inverse Simpson metrics. For beta-diversity estimation, multivariate analysis by non-metric multidimensional scaling (NMDS) with Bray-Curtis dissimilarity matrix was performed using the packages *phyloseq* and *vegan* (v2.5.7) (Oksanen *et al*., 2019).

3.7.6 RNA isolation and cDNA synthesis (Papers III, IV)

Intestinal tissue samples (30 mg each) were added to RNase-free bead beating tubes containing 1-3 mm corundum, 3 mm steel beads and 600 µL of Buffer RLT Plus (Qiagen, Germany). The samples were homogenised twice at 6000 rpm for 30 s using Precellys Evolution homogeniser (Bertin Technologies, France). RNA extraction was carried out using the RNeasy Plus Mini kit (Qiagen, Germany) according to the manufacturer's protocol. The concentration and purity of the extracted RNA were measured using NanoDrop ND-1000 (NanoDrop Technologies Montchanin, USA). RNA quality (RIN) was determined using Agilent Tapestation 4150 (Agilent Technologies, Germany). Genomic DNA contamination was removed by treating 1.2 µg of each RNA sample with RQ1 RNase-Free DNase

(Promega, USA). cDNA was synthesised using the GoScript™ Reverse Transcriptase (Promega, USA), following the manufacturer's protocol. The cDNA reaction was split so that 0.2 µg RNA was used for control without reverse transcriptase (-RT control). The cDNA samples were diluted at 1:5 ratio using nuclease-free water and stored at -20 °C until use.

3.7.7 Gene expression analysis (Papers III, IV)

Details for specific primers for qPCR of the targeted and reference genes provided in Papers III and IV. qPCR amplification of genes in each of the experimental samples was carried out in a CFX96 Touch PCR device (Bio-Rad, California, USA), using Quantitect SYBR Green (Qiagen, Germany). Each reaction was prepared in duplicate to a total volume of 25 µL per reaction, with the reaction mixture consisting of 12.5 µL Quantitect SYBR Green (2x), 1.25 µL forward primer, 1.25 µL reverse primer, 8 µL nuclease-free water and 2 µL cDNA samples as template. The thermal profile used for qPCR amplification consisted of an initial cycle of denaturation at 95 °C for 15 min, followed by 39 cycles of 95 °C for 15 s, annealing at gene-specific annealing temperature (T_a) for 30 s and extension at 72 °C for 30 s. The thermal cycle ended with melt curve analysis to verify the PCR product. The relative expression of each stress-mediated and immune-response gene was normalised with the two selected reference genes and calibrated with respect to the control samples. The efficiency of *β-actin* and *rps20* was calculated using the CFX software. The ΔΔCt value of each sample was determined by subtracting the average ΔCt value of the control from ΔCt of the test sample. Relative quantification or fold change in expression for each gene compared with the control was thus expressed as $2^{-\Delta\Delta Ct}$ (Livak & Schmittgen, 2001).

3.8 Statistical analysis

Different statistical models were used in Papers I-IV, since different experimental designs were implemented and different analytical parameters were evaluated, although some aspects were common to all papers.

In Paper I, data analysis was performed using SAS statistical software version 9.4 (SAS Institute Inc., Cary, NC, USA). The effects of experimental diet on ADC and on growth parameters (SGR, WG, FCR) were assessed by

one-way analysis of variance (ANOVA) using PROC GLM, followed by Tukey's multiple comparison test, with diet as fixed factor and tank as random factor.

In Paper II, a linear mixed effect (LME) model ("nlme" package) was used to test the effect of diets and days of feeding, and their interaction, on average OTU abundance >1%, followed by *post hoc* analysis of emmeans ("emmeans" package) (Lenth *et al*., 2019), with Tukey's multiple pairwise comparison using R statistical software version 3.6 (Pinheiro *et al*., 2014; R Core Team, 2016). Two-way analysis of similarity (ANOSIM) based on Bray-Curtis dissimilarity was performed to statistically confirm the effect of diet and day interval on beta-diversity of gut microbial composition, followed by similarity percentage analysis (SIMPER) using Paleontological Statistics Software version 4.03 (PAST).

In Paper III, the data obtained on growth parameters and gene expression were subjected to one-way ANOVA followed by Tukey's multiple comparison test using GraphPad PRISM 9.3.1.

In Paper IV, statistically significant differences between bacterial ASVs with mean abundance >1% were assessed by Kruskal-Wallis test, followed by Dunn test for *post* hoc analysis, estimated using GraphPad PRISM 9.3.1. One-way ANOSIM based on Bray-Curtis dissimilarity was performed to statistically confirm the overall differences in gut microbial composition, followed by SIMPER using PAST software. The data obtained on growth parameters, antioxidant enzyme activity and gene expression were subjected to one-way ANOVA, followed by Tukey's comparison test using GraphPad PRISM 9.3.1 for pair-wise comparisons of the different diets. The significance level was set at $p<0.05$. In addition, for the gene expression ANOVA values, which showed a distinct trend ($p\leq0.1$), an unpaired *t*-test was conducted for pair-wise comparison between prebiotic (FOS and COS) and control diets.

4. Results

4.1 Effect of dietary *Neurospora intermedia* (Papers I, II)

4.1.1 Nutritional composition and growth performance

Neurospora intermedia biomass used in the test diets in Papers I and II was found to contain 60.9% crude protein, 6.4% crude fat, 8.3% minerals (ash content) and 249.9 g kg^{-1} of nitrogen-free extract on a dry matter basis (Table 2 in Paper I). For amino acid profile see Table 2 in Paper I. The results showed no significant differences in growth parameters (WG, SGR, FCR) between the *N. intermedia* diets and the reference diet (Table 2).

Table 2. Weight gain (WG) (%), specific growth rate (% per day) (SGR) and feed conversion ratio (FCR) for the Neurospora intermedia diet (NPD), preconditioned N. intermedia diet (PD) and reference diet (RD) after the 30-day feeding trial

	RD	NPD	PD
WG (%)	45.6 ± 3.10	44.7 ± 1.50	45.1 ± 3.30
SGR (% per day)	1.25 ± 0.07	1.23 ± 0.03	1.24 ± 0.07
FCR	0.95 ± 0.05	0.96 ± 0.03	0.97 ± 0.05

4.1.2 Digestibility

Apparent digestibility coefficient for the different experimental diets fed to rainbow trout in Paper I was calculated at 10, 20 and 30-day of feeding trial. ADC for the different experimental diets and test ingredients at 30-day are presented in Table 3 and Table 4, respectively. The values obtained for the digestibility parameters in the NPD diets (dry matter, crude protein, crude fat, gross energy) were significantly higher than those in PD, while there was no significant difference between NPD and RD for dry matter and crude protein. Diet and duration of feeding had an effect on digestibility in rainbow trout (see Table 5 in Paper I).

Table 3. Apparent digestibility coefficient (ADC, %) for dry matter (DM), crude protein (CP), crude fat (CF), and gross energy (GE) in the Neurospora intermedia diet (NPD), preconditioned N. intermedia diet (PD) and reference diet (RD) during the 30-day feeding trial

	RD	NPD	PD
DM	78.5 ± 0.5^{a}	78.4 ± 0.4^{a}	76.8 ± 0.4^{b}
CP	92.8 ± 0.2^{a}	92.4 ± 0.2^{a}	91.6 ± 0.3^{b}
CF	90.9 ± 0.6^{b}	93.0 ± 0.6^{a}	92.3 ± 0.4^{a}
GE	84.0 ± 0.4^{a}	82.8 ± 0.4^{b}	81.3 ± 0.4^{c}

Values within rows with different superscripts letters are significantly different ($p<0.05$)

Table 4. Apparent digestibility coefficient (ADC, %) of test ingredient for dry matter (DM), crude protein (CP), crude fat (CF), and gross energy (GE) during the 30-day feeding trial

	NPD	PD
DM	78.5 ± 1.1	78.2 ± 1.5
CP	92.8 ± 0.5^{a}	91.6 ± 0.6^{b}
CF	98.0 ± 1.8	95.7 ± 1.2
GE	80.4 ± 1.5^{a}	75.2 ± 1.3^{b}

Values within rows with different superscript letters are significantly different ($p<0.05$).

4.1.3 Modulation of gut microbiota

Principal coordinate analysis (PCoA) based on Bray-Curtis index demonstrated a shift in gut bacterial composition in fish fed the different diets from day 0 to day 30 (Figure 5). The effect of diet was easily observed at 10 to 20 days. Percentage variation (PoV) explained for axis 1 and 2 while using Bray-Curtis index, were 25.5% and 10.3%, respectively. The results of statistical analysis and interaction plots investigating the effect of treatments within and between 10-day intervals of feeding on the abundance of two OTUs are shown in Figure 6. Diet and duration of feeding modulated the gut microbiota of rainbow trout, with an increase in abundance of *Lactococcus* from day 0 to day 30 (Figure 6A) and a decrease in abundance of *Peptostreptococcus* over the same period (Figure 6B).

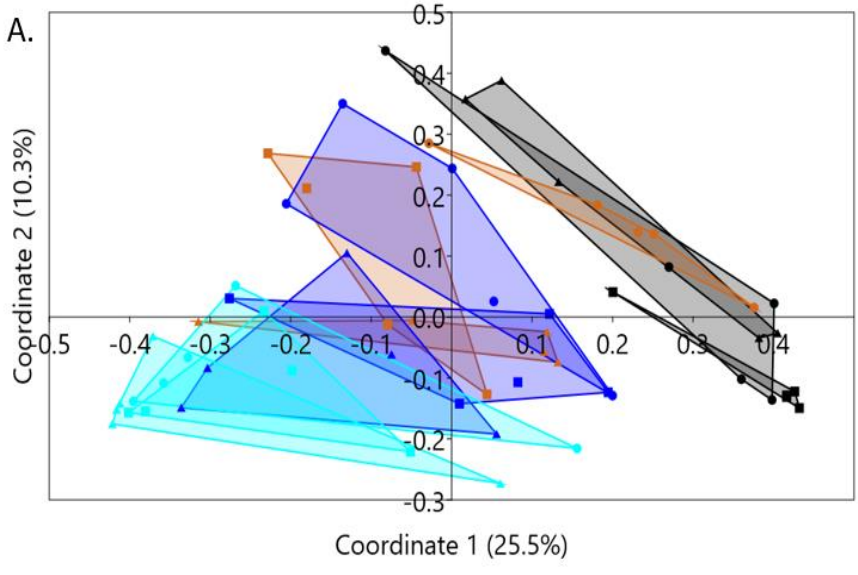


Figure 5. Principal coordinate analysis plot based on Bray-Curtis index showing the shift in gut bacterial community of rainbow trout with diet and time for the: reference diet (●), preconditioned diet (■) and non-preconditioned diet (▲). Different colours represent 10-day intervals: days 0 (black), 10 (orange), 20 (blue) and 30 (cyan).

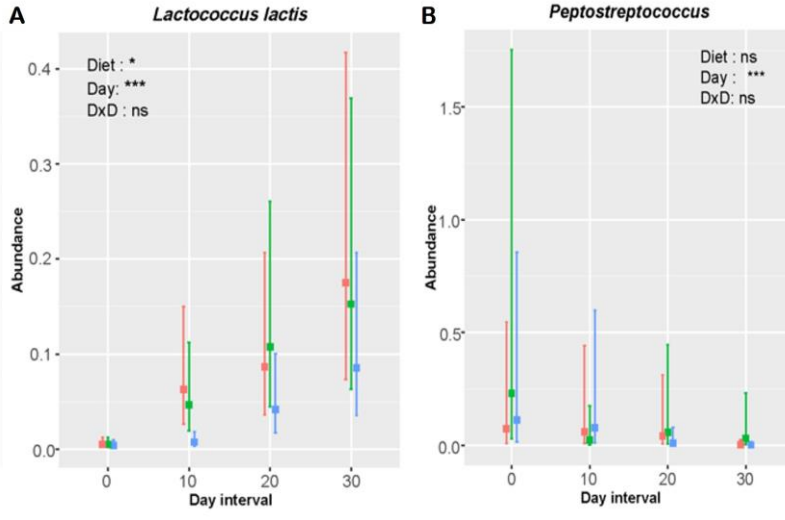


Figure 6. Interaction plot showing relative abundance of (A) *Lactococcus* and (B) *Peptostreptococcus* in the gut bacterial community of rainbow trout fed different diets (reference diet: blue, preconditioned diet: green, non-preconditioned diet: red).The vertical bars in the plot represent confidence interval and overlapping bars mean that differences are not significant. Significance: ***p<0.001, *p<0.05, ns p>0.05.

4.2 Effect of dietary *Yarrowia lipolytica* (Paper III)

4.2.1 Fish performance

Percentage weight gain (p=0.082), SGR (p=0.055) and FCR (p=0.310) of diets containing *Y. lipolytica* did not differ significantly from the control after 45 days of feeding (Table 5). No differences in HSI (p=0.197), GSI (p=0.389) or CF (p=0.899) were observed between the experimental diets. Biometric indices were within the range 1-1.6, indicating overall good health status of the fish (Table 5).

Table 5. *Growth parameters and performance indices for rainbow trout fed different experimental diets containing 2% or 5% whole (WY) or autolysed (AY)* Yarrowia lipolytica *for 45 days*

	Experimental diet				
	Control	2%-WY	5%-WY	2%-AY	5%-AY
WG	94.6 ± 6.2	96.8 ± 8.2	120.8 ± 7.3	107.4 ± 7.8	113.6 ± 8.6
SGR	1.18 ± 0.06	1.17 ± 0.08	1.41 ± 0.06	1.29 ± 0.06	1.33 ± 0.07
FCR	0.83 ± 0.08	0.85 ± 0.11	0.64 ± 0.02	0.74 ± 0.07	0.70 ± 0.08
HSI	1.33 ± 0.04	1.51 ± 0.05	1.46 ± 0.14	1.41 ± 0.05	1.61 ± 0.09
GSI	1.05 ± 0.07	1.21 ± 0.06	1.16 ± 0.04	1.13 ± 0.03	1.16 ± 0.07
CF	1.22 ± 0.04	1.21 ± 0.00	1.24 ± 0.01	1.19 ± 0.02	1.22 ± 0.04

Values shown are mean ± SE. One-way ANOVA was performed to identify differences between experimental diets.

4.2.2 Modulation of gut microbiota

Alpha- and beta-diversity of gut microbiota showed no differences between the *Y. lipolytica* and control diets (see Figure 1 in Paper III). Log fold change (LFC) analysis was carried out to compare positive and negative associations of microbial communities with the *Y. lipolytica* diets and with the control diet (Figure 7). *Desulphovibrionaceae* showed a synergistic association between the control and WY diets. *Sphingobactericaceae* (LFC ~4.5, p<0.05) was positively linked with the 5%-WY and 5%-AY diets, while *Rhodobacteraceae* (LFC ~3, p<0.05) showed a positive association with the 2%-WY and both AY diets.

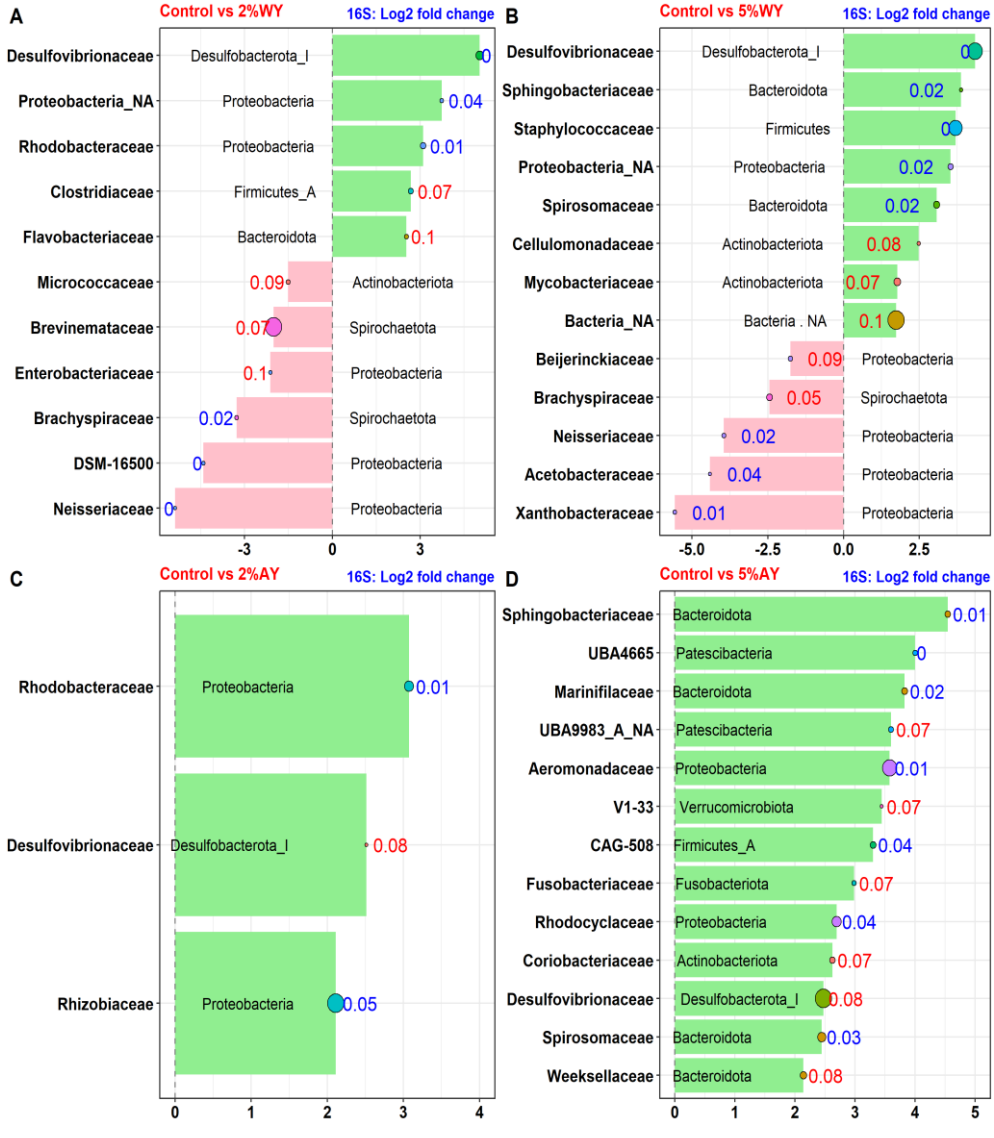
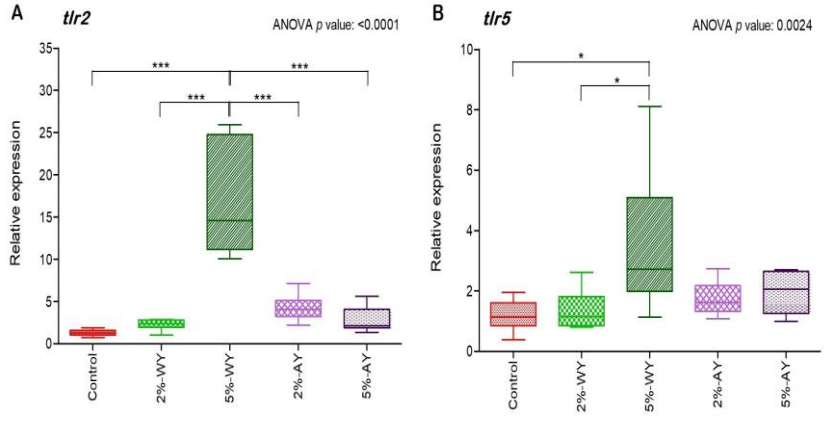


Figure 7. Log2 fold change analysis (LFCa) plot of differential abundance of microbial families in rainbow trout fed diets containing 2% or 5% whole (WY) or autolysed (AY) *Yarrowia lipolytica*. Positive and negative Log2 fold change represents a synergistic and inhibitory effect, respectively, on the microbial community at family level due to diet. (A) Control vs. 2%-WY, (B) Control vs. 5%-WY, (C) Control vs. 2%-AY, and (D) Control vs. 5%-AY. Numerical values beside bubbles indicate *p*-value for differential abundance (blue $p<0.05$, red $p>0.05$).

4.2.3 Effects on immune gene expression in gut

Immunological responses at molecular level revealed by mRNA expression analysis of immune-relevant genes in the distal intestine are shown in Figure 8. The expression profile of immune genes demonstrated that *tlr2* (Figure 8A), *tlr5* (Figure 8B), *c3* (Figure 8C) and *c-type lectin* (Figure 8D) gene transcripts were significantly upregulated ($p<0.05$) in fish fed the 5%-WY diet compared with the other diets. In addition, the analysis showed numerically very high (~15- to 54-fold change) and statistically significant ($p<0.0001$) upregulation in *igt* gene transcript (Figure 8E) for all the yeast diets (whole or autolysed) compared with the control diet. For *cd4* gene transcript (Figure 8F), only 5%-WY fish showed significant ($p<0.05$) upregulation, with ~3-fold change.



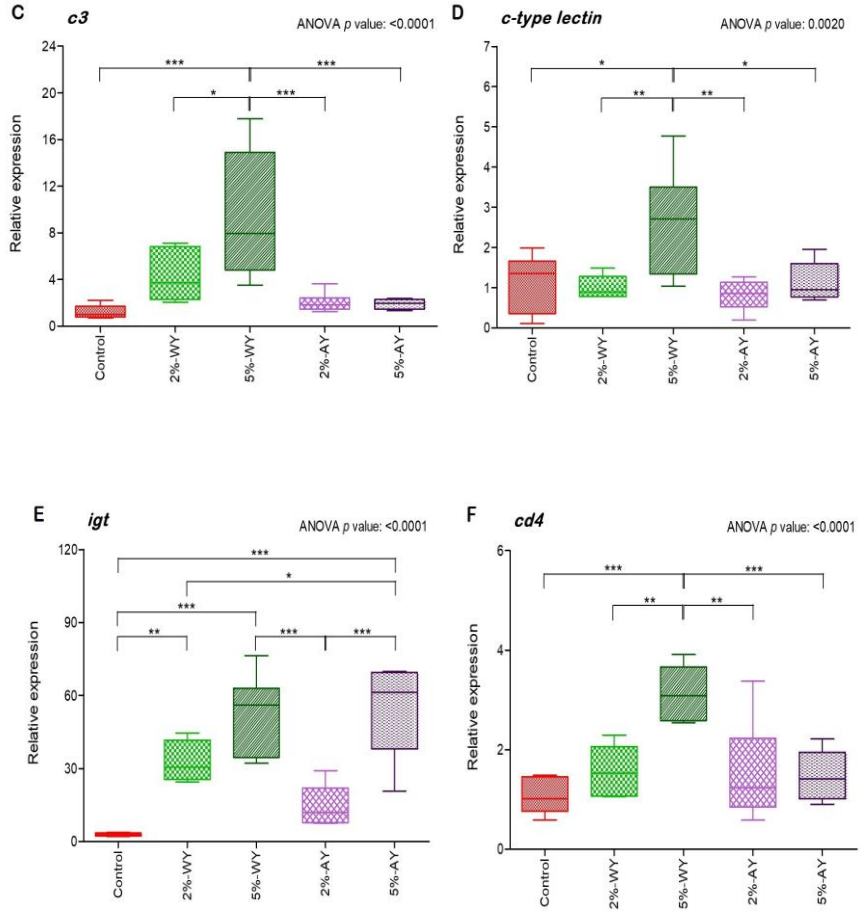


Figure 8. Relative expression of immune-related genes in intestine tissue of rainbow trout fed the control diet and diets containing 2% or 5% whole (WY) or autolysed (AY) *Yarrowia lipolytica* (2%-WY, 5%-WY, 2%-AY and 5%-AY) for 45 days. Expression levels of each gene were compared between the experimental diets relative to the naïve control. Relative expression level of the genes (A) *tlr2*, (B) *tlr5*, (C) *c3*, (D) *c-type lectin*, (E) *igt*, and (F) *cd4*, plotted as individual and mean (n=6) fold changes in transcript level. One-way ANOVA was performed to determine significant ($p<0.05$) differences between diets, followed by Tukey's multiple comparison test (*$p<0.05$; **$p<0.01$, ***$p<0.001$). (*tlr*: toll-like receptors, *c3*: complement 3, *igt*: immunoglobulin T, *cd4*: cluster of differentiation 4.)

4.3 Effect of dietary cello-oligosaccharides (Paper IV)

4.3.1 Fish performance

Growth performance of rainbow trout fed diets containing FOS (0.5%) or COS (0.1%, 0.5% and 1.5%) or a control diet is shown in Table 6. There were no significant (p>0.05) differences between the diets in terms of weight gain percentage (WG %), specific growth rate (SGR) or feed conversion ratio (FCR).

Table 6. *Growth parameters for rainbow trout fed a control diet and diets containing fructo-oligosaccharides (FOS 0.5%), and cello-oligosaccharides (COS 0.1%, COS 0.5%, COS 1.5%) diets for 45 days*

Experimental diet	Growth parameter		
	WG (%)	SGR	FCR
Control	131.8 ± 10.9	1.77 ± 0.09	0.84 ± 0.03
FOS 0.5%	137.9 ± 9.5	1.85 ± 0.08	0.82 ± 0.01
COS 0.1%	144.3 ± 11.6	1.89 ± 0.09	0.78 ± 0.02
COS 0.5%	123.9 ± 8.8	1.72 ± 0.08	0.90 ± 0.08
COS 1.5%	142.7 ± 11.8	1.87 ± 0.09	0.78 ± 0.02
	p value: 0.648	*p* value: 0.639	*p* value: 0.282

Values shown are mean ± SE. One-way ANOVA was performed for each parameter and *p*-values are shown in the respective column.

4.3.2 Modulation of gut microbiota

Multivariate analysis of microbiota taxa composition using principal component analysis (PCA) showed a clear distinction in overall microbial composition for COS 1.5% compared with the control and FOS 0.5% and other COS diets (Figure 9) (for ANOSIM data, see Paper III). Effects of the different COS and FOS diets on the abundance in microbial community also emerged at family level, where the bacterial families *Brevinemataceae* (p=0.020), *Ruminococcaceae* (p=0.018), *Chitinobacteraceae* (p=0.039), *Bacillaceae* (p=0.001) and *Lactobacillaceae* (p=0.044) differed significantly in abundance between the diets (Figure 10).

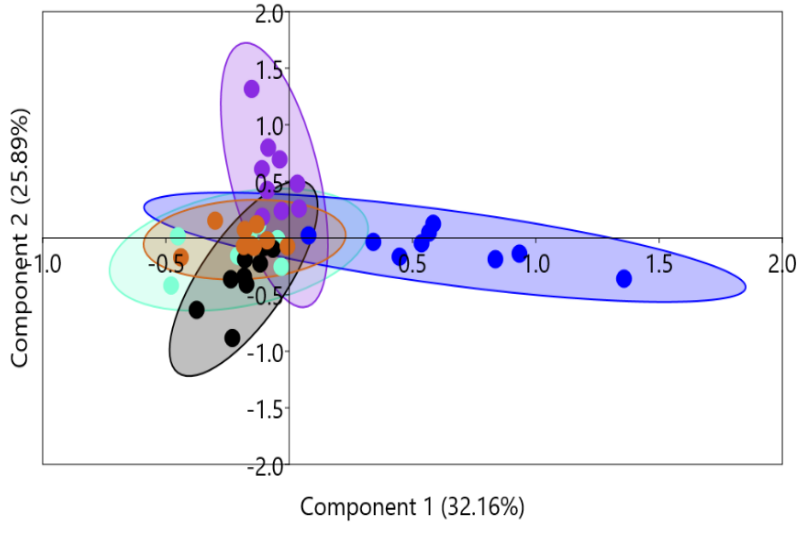


Figure 9. Principle component analysis of gut microbial communities in rainbow trout fed with different experimental diets – Control (●), FOS 0.5% (●), COS 0.1% (●), COS 0.5% (●), and COS 1.5% (●).

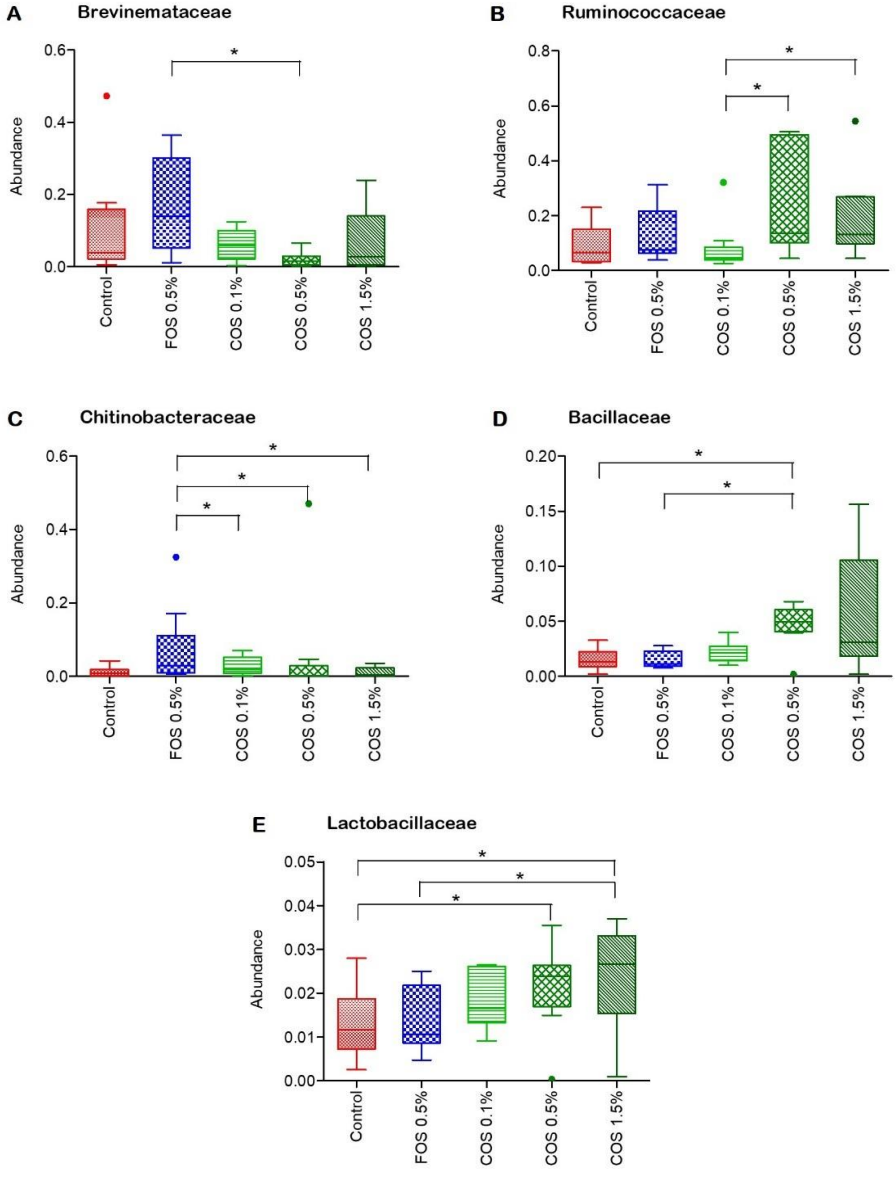


Figure 10. Comparison of gut microbial community abundance at family level in rainbow trout fed different experimental diets containing fructo-oligosaccharides (FOS), and cello-oligosaccharides (COS) for 45 days : Control, FOS 0.5%, COS 0.1%, COS 0.5% and COS 1.5%. (A) *Brevinemataceae*, (B) *Ruminococcaceae*, (C) *Chitinobacteraceae*, (D) *Bacillaceae* and (E) *Lactobacillaceae*. Values presented as box and whiskers plot, with line inside the box representing median value. Significant differences (p<0.05) in microbial community between the diets are indicated by asterisks (*). Outliers are represented by dots with the respective diet colour.

4.3.3 Effects on antioxidant activity

The effects of the COS and FOS diets on antioxidant capacity (superoxide dismutase, *sod*; catalase, *cat*; glutathione peroxidase, *gpx*) in rainbow trout were assessed using comparative mRNA expression in the distal intestinal tissue (Figure 11) and downstream enzyme activities in blood serum (Table 7). Among these, *sod* showed a significant difference ($p<0.05$) at both mRNA and enzyme level.

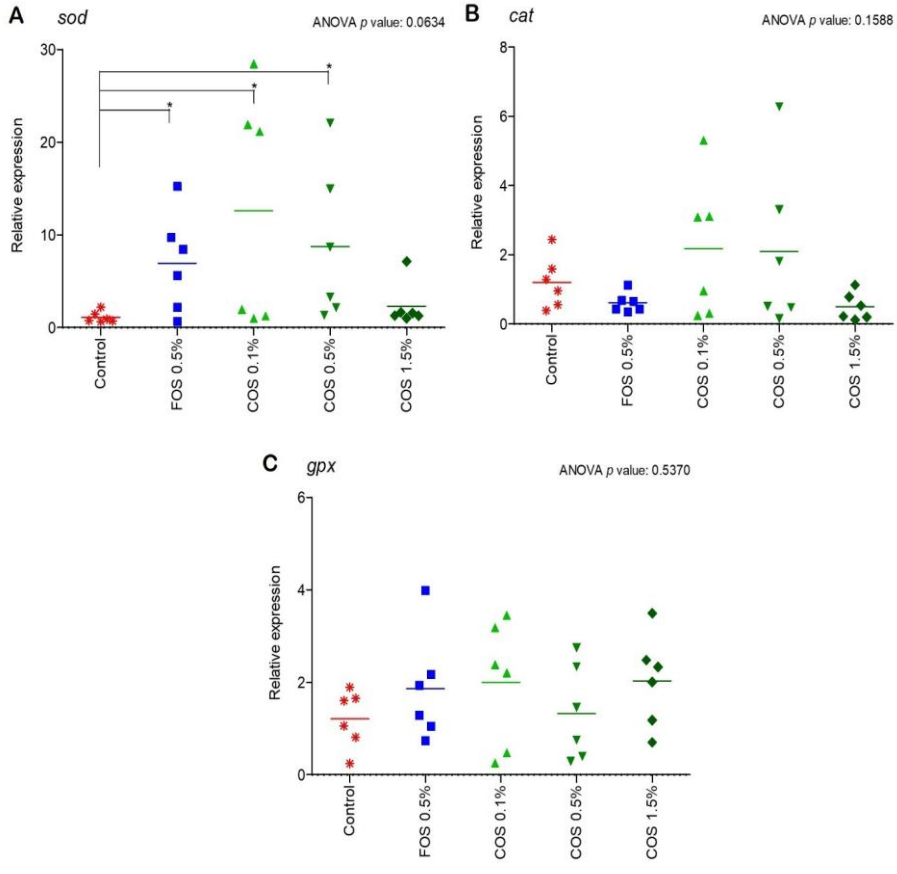


Figure 11. Relative expression of oxidative stress-related genes, (A) *sod*, (B) *cat*, and (C) *gpx* in gut tissue of rainbow trout fed different experimental diets for 45 days: Control, FOS 0.5%, COS 0.1%, COS 0.5% and COS 1.5%. Expression levels of each gene for each diet were plotted as individual and mean fold change in gene transcript level. Significant differences between diets (one-way ANOVA) are indicated by *p* value ($p<0.05$) and *p* value ≤ 0.1 considered to indicate a tendency. Differences between COS or FOS diets and the control (unpaired *t*-test) are indicated by asterisks (*$p<0.05$).

Table 7. Antioxidant activity in serum of rainbow trout fed a control diet and diets containing fructo-oligosaccharides (FOS 0.5%) and cello-oligosaccharides (COS 0.1%, COS 0.5%, COS 1.5%) for 45 days

Experimental diet	Superoxide dismutase, SOD (U/mL)	Catalase, CAT (U/mL)	Glutathione peroxidase, GPx (U/mL)
Control	20.2 ± 5.2^{a}	47.1 ± 2.1	274.4 ± 54.4
FOS 0.5%	49.1 ± 4.5^{b}	38.9 ± 3.9	288.1 ± 65.3
COS 0.1%	44.5 ± 7.4^{b}	57.8 ± 2.9	328.5 ± 73.9
COS 0.5%	52.9 ± 5.7^{b}	39.3 ± 9.2	364.3 ± 84.7
COS 1.5%	37.7 ± 3.5^{b}	39.4 ± 1.2	132.7 ± 36.9
	p-value: 0.012	*p*-value: 0.319	*p*-value: 0.198

Values shown are mean ± SE. One-way ANOVA was performed for each parameter and *p*-values are included in the respective column. Values within columns with different superscript letters, obtained by Tukey's multiple pairwise comparison, are significantly different ($p<0.05$).

4.3.4 Effects on immune gene expression in intestine

The results showed a distinct trend ($p\leq0.1$) in the expression of *tlr2* (Figure 12A) gene transcript (~2-5 fold) in fish fed COS diets compared with the control. The difference in gene expression was not statistically significant, likely due to wide variation in the expression pattern in individual fish within each diet. The *c3* gene (Figure 12B) showed significant upregulation ($p<0.05$) in fish fed the COS diets compared with the control. However, the *c-type lectin* (Figure 12C) gene demonstrated distinct tendency ($p\leq0.1$) in the transcript expression in COS diets, but on pairwise comparison with the control diet, only the COS 0.5% diet showed statistical difference ($p<0.05$).

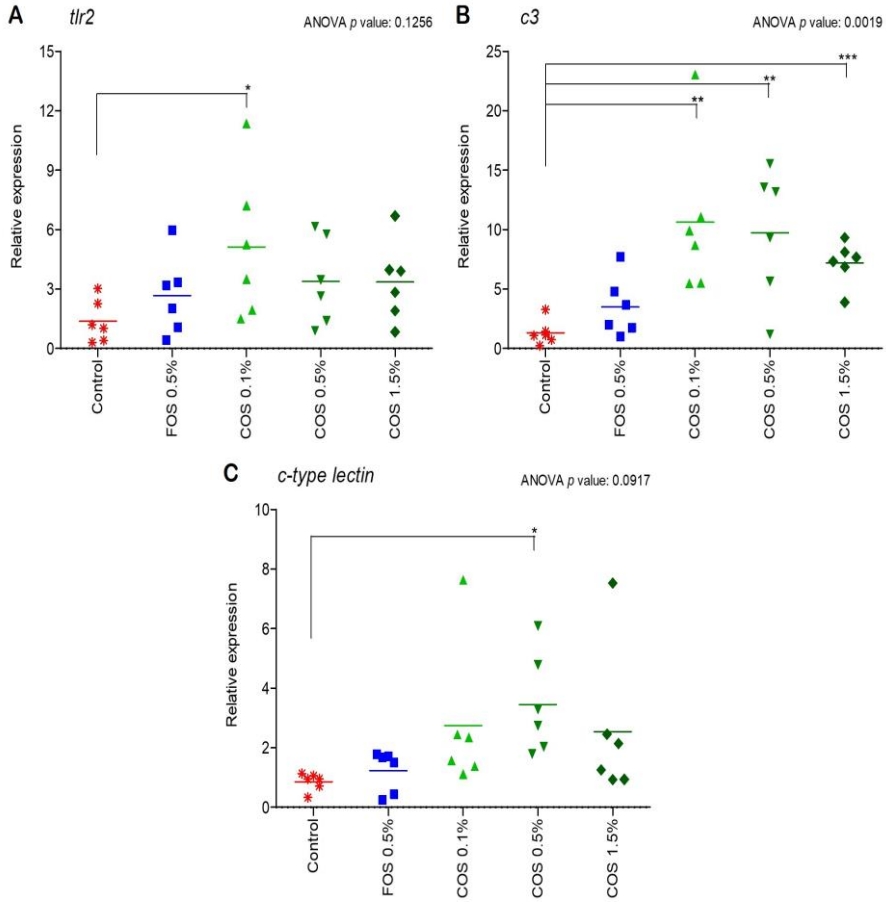


Figure 12. Relative expression of immunity related genes, (A) *tlr2*, (B) *c3*, and (C) *c-type lectin* in gut tissue of rainbow trout fed different experimental diets for 45 days: Control, FOS 0.5%, COS 0.1%, COS 0.5% and COS 1.5%. Expression levels of each gene for each diet were plotted as individual and mean fold change in gene transcript level. Significant differences between diets (one-way ANOVA) are indicated by *p* value ($p<0.05$) and *p* value ≤ 0.1 considered to indicate a tendency. Differences between COS or FOS diets and the control (unpaired *t*-test) are indicated by asterisks ($*p<0.05$).

5. Discussion

In this thesis, separate experiments were conducted to evaluate the potential effect of three alternative feed ingredients (*Neurospora intermedia*, *Yarrowia lipolytica* and cello-oligosaccharides) in the diet of rainbow trout with respect to digestibility and/or growth, gut microbiota modulation and mucosal immune response in the intestine. Important results from these studies are discussed in this chapter. All results from all studies are discussed in detail in Papers I-IV.

5.1 Dietary *Neurospora intermedia* – fish health

Fungal biomass has been identified as a potential protein-rich alternative to fishmeal in aquafeed (Ferreira *et al*., 2016; Karimi *et al.*, 2021; Sar *et al*., 2022). In this thesis, biomass of *N. intermedia*, belonging to the filamentous fungi, was tested as a partial substitute for fishmeal in the diet of rainbow trout. Analysis of the nutritional profile (dry mass basis) of *N. intermedia* biomass revealed that it contained 60.9% crude protein, 6.4% crude fat, 8.3% ash (minerals) and 249.9 g kg^{-1} of nitrogen-free extract. However, amino acid analysis showed that the dried fungal biomass used in the test diets had an actual protein content of 47.1%. It also revealed that *N. intermedia* biomass contained significant amounts of indispensable amino acids and dispensable amino acids. Indispensable amino acids made up 48.1% of the total amino acid content, which is even higher than in fishmeal (47.0%) and soyabean meal (45.9%) currently used in conventional aquafeeds (NRC, 2011). This confirms the potential of *N. intermedia* biomass as an important alternative protein source for aquafeed.

Feed processing involving heat treatment has been found to improve the degree of gelatinisation of starch present in feed ingredients, which can enhance pellet quality, stability, palatability and digestibility (Romano & Kumar, 2019). Microbial cell walls contain complex molecules such as chitin, glucan and mannans, which restrict nutrient utilisation, leading to suggestions that heat processing could improve nutrient utilisation and animal performance by modulating gut microbiota (Windell *et al*., 1978). Therefore, in this thesis (Paper-I & -II), after preparation of *N. intermedia*-based feed by cold-pelleting technology, one-half of the feed was subjected to preconditioning by heat treatment (105 ºC for 5 min) to increase the degree of gelatinisation and emulate temperature treatment under extrusion conditions. In order to evaluate the optimize condition for *N. intermedia*-included diets, we conducted a 30-day feeding trial with the two test (PD and NPD) and control (RD) diets in rainbow trout.

5.1.1 Effects on fish performances

The results showed that inclusion of *N. intermedia* fungal biomass in the diets had positive, but non-significant, effects on growth parameters in the fish (WG, SGR, FCR) compared with the control diet. In line with the general recommendation of higher SGR and lower FCR for ensuring good fish performance (Abdel-Aziz *et al*., 2021), the results showed better SGR (1.23-1.24) and FCR (0.96-0.97) as compared to another fungal (*Rhizopus oryzae*) biomass supplemented diets in Arctic char (SGR 0.97, FCR 9.01) (Vidakovic *et al*., 2016).

5.1.2 Effects on digestibility

Consistent with the growth data, the results showed high and comparable ADC of the *N. intermedia* diets with respect to the fishmeal-based control diet, and are superior than that observed in *R. oryzae*-supplemented diets for Arctic char (Vidakovic *et al.*, 2016). Although, the ADC for CP was marginally lower than the RD diet but it was in the suitable range to determine the high digestibility of the fungal diets. Further, the increased CF digestibility observed for NPD and PD diets as against RD diet may be explained by the lower energy density and fat content in the fungal diets. Moreover, the results demonstrating higher values for various ADC parameters (CP: 92.4%, CF: 93%, and GE: 82.8%) for NPD diet with respect

to PD diet (CP: 91.6%, CF: 92.3%, and GE: 81.3%) indicates no beneficial effect of heat-treatment of the fungal feed on digestibility. Also, it is to be mentioned here that despite having high ADC, the recipient fish were healthy throughout the feeding trial, whereas, there was reported diarrhoea incidence in Arctic char fed with *R. oryzae*-supplemented diets (Langeland *et al*., 2016), thereby indicating for the suitability of *N. intermedia* in fish diets.

5.1.3 Effects on gut microbiota over time

While diet is an important mediator of gut microbial diversity, the potential role of thermal processing associated with feed preparation in shaping gut microbiota has not been explored previously, other than the effect of shifting the diet alone. Information on the effect of heat processing of feed on the fish gut microbiome is scarce. In this thesis, gut microbial analysis was carried out in rainbow trout fed a commercial diet containing fishmeal as a major source of protein during a 14-day acclimatisation period until day 0 and then diets with *N. intermedia* until day 30. The results showed no differences in overall microbial composition between the preconditioned and non-preconditioned *N. intermedia* diets at day 30. However, Zhang & Li (2018) observed a decrease in gut microbiota at taxonomic and OTU levels when catfish were fed with steam-processed (for 15 min; highest temperature 100 ºC for 2-3 min) feed compared with non-processed feed. Thus, further studies are needed to understand this correlation between microbiota and heat-processed diet.

There was a gradual shift in bacterial communities between fish fed the commercial diet (day 0) and those fed the experimental diets (day 10-30), with diet and time both having an effect in shaping the gut communities. Previous studies have also reported a change in gut microbiota after first feeding following a change in diet for salmon, rainbow trout and brown trout (*Salmo trutta*) (Ingerslev *et al*., 2014; Michl *et al*., 2017, 2019). Michl *et al*. (2017) observed no change in gut microbiota over time after a certain point, or with longer feeding duration with the same diet, and concluded that microbiota composition depends largely on the actual diet fed at the time of sample collection. In this study, the gut microbiota differed significantly at day 10 and day 20, but was similar at day 30 irrespective of diet, suggesting that the microbiota prevailing at this time point was not influenced by heat

processing of the diet or by *N. intermedia* inclusion. However, temporal changes were seen for all diets from day 20 to day 30. Early differences in microbial composition (up to day 20) may reflect adaptation to the changed environment due to a dietary intervention. Longer studies are needed to confirm whether there is any further change in microbial composition after day 30. Analysis of the temporal microbiota data at 10-day intervals revealed that *Peptostreptococcus* was dominant at day 0 but, over time, abundance of *Lactococcus* prevailed, which can be attributed to the change in substrate. *Peptostreptococcus* is generally present in high abundance in protein-rich environments, and plays an important role in amino acid catabolism and absorption in the gut (Davila *et al*., 2013; Neis *et al*., 2015). The abundance of one taxon can suppress that of another, depending on nutrient availability for growth. A shift in microbial composition from *Streptococcus* to *Lactobacillus* has been reported in Atlantic salmon fed fishmeal-free diets or diets with fishmeal replaced with plant protein (Hartviksen *et al*., 2014).

The increase in abundance of *Lactococcus* in both fungal-based test diets and fishmeal-based control diet is in accordance with previous studies where, rainbow trout fed plant protein-based diet and Atlantic salmon fed fishmeal-free diet showed increase in abundance of *Lactobacillales* (Schmidt *et al*., 2016; Michl *et al*., 2017). A study using PCR-TTGE-dependent bacterial quantification also showed an increase in *Lactobacillus* and *Lactococcus* in Atlantic salmon fed fermented soymeal (30% replacement of fishmeal) diets (Catalán *et al*., 2018). Therefore, it is possible that components in the cell wall of *N. intermedia*, such as β-glucan, chitin and glycoproteins, act as fermentable substrate for *Lactobacillales* which in turn resulted in their overall abundance in the test diets along with fishmeal-based control diet. Increased *Lactobacillales* abundance has been observed in yeast diet fed Artic char and rainbow trout (Huyben *et al*., 2017; Nyman *et al*., 2017). *Lactococcus lactis* are natural inhabitants of the fish gut and have the ability to adhere and colonise (Seppola *et al*., 2006; Gatesoupe, 2008). Studies have shown that use of *L. lactis* when used as probiotic, can enhance weight, immunity and disease resistance, improve the gut architecture and modulate the intestinal microbial composition in fish (Heo *et al*., 2013; Xia *et al*., 2018; Xia *et al*., 2019; Won *et al*., 2020). Thus, the abundance of *L. lactis* positive influence of *N. intermedia* on gut immunity and integrity, although further research is required to draw conclusion.

5.2 Dietary *Yarrowia lipolytica* – fish health

Yeasts are considered as the most enriched alternative feed resources due to their relatively high protein, energy, nucleic acid and micronutrient content (Yuan *et al*., 2019; Rimoldi *et al*., 2020). In addition, the presence of immune-stimulating compounds, such as β-glucan, chitin and mannan-oligosaccharides, make yeasts the preferred functional ingredient for aquafeeds (Torrecillas *et al*., 2014; Pongpet *et al*., 2016; Gong *et al*., 2019). However, the bioactivity of these cell wall components depends on the yeast species, the fermentation conditions during yeast production and the downstream processing used before incorporating them into a salmon diet (Øverland & Skrede, 2017). For better utilisation of yeast-derived nutrients and higher acceptance by the recipient fish, several studies prescribe use of yeast extract or autolysis of yeast cells before dietary inclusion (Berto *et al*., 2016; Sönmez, 2017; Hoshino *et al*., 2020; Rimoldi *et al*., 2020). Downstream processing of the yeast leads to breakdown of insoluble macromolecules such as proteins and nucleic acids into soluble peptides, amino acids and nucleotides, which assists in modulation of gut microbial activity in the host fish (Agboola *et al*., 2022). However, contradictory views have also been expressed, *e.g*. it is reported that heat treatment can change the molecular weight or configuration of immunogenic compounds (*e.g*. β-glucans) and thereby alter their bioactivity (Kaur *et al*., 2019; Agboola *et. al*., 2021; Zheng & Huang, 2022). The comparative study in Paper-III in this thesis evaluated whole and autolysed *Y. lipolytica*, incorporated in the diet at 2% and 5% levels, in terms of gut microbiota and immuno-modulatory capacity in rainbow trout.

5.2.1 Effects of downstream processed yeast on fish performance

Percentage weight gain in the experimental diet groups ranged from 94-120%, but there were no significant differences (p=0.082) between the yeast-fed fish and the control after the 45-day feeding period. Consequently, no significant differences (p>0.05) in SGR and FCR were seen for the diets with *Y. lipolytica*. These results are in line with previous findings in a study involving dietary supplementation of *Y. lipolytica* in Atlantic salmon, where there was no significant effect of the yeast on growth performance (Hatlen *et al*., 2012). However, Neuls *et al*. (2021) observed improved growth

performance in Nile tilapia fed diets with 3-6% *Y. lipolytica*. In the study, HSI, VSI and CF ranged between 1 and 1.6, demonstrating good overall health status of the fish. However, no significant differences (p>0.05) in these biometric indices were observed between the experimental diets.

5.2.2. Effects of processed yeast on modulation of faecal microbiota

As mentioned above, it was assumed that downstream processing for whole and autolysed *Y. lipolytica* might have different impacts on the nutritional and cell wall composition of the yeast, and thereby on gut microbiota composition between the diet groups. However, both alpha-diversity (Shannon and Simpson index) and beta-diversity (Bray-Curtis index) showed no differences between the diets. Similar results have been reported for Arctic char, with no differences in bacterial richness and diversity in the gut microbiota when 40% of the fishmeal in the diet was replaced by intact or extracted yeast cells (Nyman *et al*., 2017).

Log fold change analysis (LFCa) showed positive association of *Desulphovibrionaceae* with the whole yeast diets at 2% and 5% levels. Both the WY and AY diets at 5% level demonstrated a positive association for *Sphingobactericaceae*, and at 2% inclusion level *Rhodobacteraceae* was found to be the dominant microbial family. The positive association of *Sphingobactericaceae* with the 5%-WY and 5%-AY diets can relate to the ability of the bacteria to grow on yeast lipid substrate with production of bioactive sphingolipid and inositol, important regulators in metabolic pathways (Hannun *et al*., 2018; Johnson *et al*., 2020; Heaver *et al*., 2022). Further, there was dominance of *Staphylococcaceae* in the 5%-WY fish, although their presence in the gut did not cause harm to the host fish, as evident from the normal growth and 100% survivability. Previous studies have also observed augmentation of *Staphylococcaceae* in gut microbiota of Atlantic salmon fed different dietary supplements (Askarian *et al*., 2012; Abid *et al*., 2013).

5.2.3. Immunomodulatory effects of the processed yeast

As there were non-significant impacts of whole or autolysed yeast on growth performance and gut microbiota, it was not possible to determine the form and concentration of *Y. lipolytica*, that would be most beneficial for the fish. For this reason, we studied the immunomodulatory effects of the yeast

diets in the distal intestine, an important site for antigen sensing and uptake by its enterocytes (Rombout *et al*., 2011).

Microbial associated molecular patterns (MAMPs), such as β-glucan and α-mannan derived from yeast cell walls, have immunomodulatory effects in fish (Guerreiro *et al*., 2018; Rawling *et al*., 2021). These MAMPs are reported to activate different PRRs present on innate immune cells that effectively allow the host to determine the immune fate of localised GALT. In teleost, *tlrs* and *c-type lectins* families are important in recognition of yeast and its derivatives, such as mannans and β-glucans (Patin *et al*., 2018; Petit *et al*., 2020). Thus, the significantly higher ($p<0.05$) upregulation (~2-fold) of *tlr2* (~13-fold), *tlr5* (~3-fold), *c3* (~4-fold) and *c-type lectin* gene transcripts in the fish fed 5%-WY indicates structural stability and antigenicity of whole yeast-derived MAMPs compared with the autolysed form or lower inclusion levels.

Similarly, the higher level (~3-fold) of *cd4* gene transcript in the 5%-WY diet may suggest that the whole yeast was more efficient in presenting antigenic motifs to $CD4^+$-T-cells for recognition, binding and processing (Hoare *et al*., 2022). However, the positive correlation between the *igt* gene transcript (~15- to 54-fold change) and the concentration of *Y. lipolytica* in the diet indicates ability of yeast-derived β-glucan/mannans to act as a powerful antigen to stimulate B-cells for immunoglobulin (*igs*) production, irrespective of structural conformation of the yeast cells owing to autolysis. Similar upregulation of immunoglobulin gene transcripts has been observed in rainbow trout intestine following dietary supplementation with β-glucan (Porter *et al*., 2023).

Thus, from the overall gene expression results, it can be suggested that whole *Y. lipolytica* added at ≥5% level to the feed of rainbow trout has a greater immunomodulatory effect in the recipient fish than autolysed *Y. lipolytica* at a similar inclusion level.

5.3 Dietary cello-oligosaccharides (COS) – fish health

Several oligosaccharides are already widely applied in aquaculture, *e.g*. oligofructose, xylo-oligosaccharides, fructo-oligosaccharides, mannan-oligosaccharides, galacto-oligosaccharides, to improve disease resistance *via* modulation of gut microbiota and improved gut physiology (Mussatto & Mancilha, 2007; Wang e*t al*., 2010; Akrami *et al*., 2013); enhancement of growth and metabolic activity (Ortiz *et al*., 2013; Zhang *et al*., 2014); and orchestration of protective immune responses (Guerreiro *et al*., 2016). The effect of functional prebiotics largely depends on their inclusion level in diets, size of fish, type and dose, duration of feeding and rearing conditions (Song *et al*., 2014; Hoseinifar *et al*., 2019; Dawood *et al*., 2020). The stability of oligosaccharides depends on the sugar residue content, ring form, anomeric configuration and linkage types (Raman *et al*., 2005). Cello-oligosaccharides have some particular physico-chemical, rheological and functional properties which enable their use to improve dispersions and rheological properties of the materials and/or formulations to which they are incorporated (Kluge *et al*., 2019; Ávila *et al*., 2021). In Paper-IV of this thesis, rainbow trout were fed with COS diets at graded levels to investigate its biological significance.

5.3.1 Effects of COS on growth parameters

Analysis of growth parameters suggested that the fish were performing well and healthy. It was expected that incorporation of the prebiotic would enhance the biological responses in terms of better resistance to pathogens, modulation of microbial composition or immune gene expression, as compared to impacting growth parameters. Previous studies of oligosaccharides-based prebiotics in diets also found no effect on growth performance of Gulf sturgeon (*Acipenser oxyrinchus*) (Pryor *et al*., 2003), turbot (*Scophthalmus maximus*) (Mahious *et al*., 2006), hybrid tilapia (*Oreochromis niloticus*) (Genc *et al*., 2007), Atlantic salmon (Grisdale-Helland *et al*., 2008) or sea bream (*Sparus aurata*) (Guerreiro *et al*., 2016).

5.3.1 Effects of COS on faecal microbiota

The faecal microbiota analysis showed the effects of the different COS and FOS diets on the abundance in microbial community at family level. The

bacterial families *Brevinemataceae*, *Ruminococcaceae*, *Chitinobacteraceae*, *Bacillaceae* and *Lactobacillaceae* differed significantly between fish fed with the COS and FOS diets as compared to control. *Ruminococcaceae*, which is associated with production of short-chain fatty acids by fermentation of ingestible carbohydrates such as resistant starch or dietary fibre (Rimoldi *et al*., 2020), was found to be the most abundant family in the COS 0.5% fed fish. However, *Ruminococcaceae* are more common in human gut environment (Walker *et al*., 2011; Ze *et al*., 2013), where microbes live at higher temperature and have longer gut transit time than in fish. As this study did not quantify short-chain fatty acids in the gut, so it would be interesting to confirm the association of *Ruminococcaceae* with COS-derived metabolite production as future research. Further, the study showed relatively higher abundance of *Bacillaceae* and *Lactobacillaceae* in the diets with COS compared with FOS, which is in agreement with findings by Ortiz *et al*. (2013) where, FOS fermentation in the gut of rainbow trout did not affect short-chain fatty acid and/or lactic acid production, and in turn resulted in lower abundance of LAB.

5.3.2 Effects of COS on antioxidant activity

Free radicals are highly reactive molecules that can damage cells and tissues, leading to oxidative stress and inflammation. Fish, like other animals, produce free radicals as a natural part of their metabolism, but they also face additional sources of oxidative stress, such as exposure to infectious agents and dietary or environmental stress. Antioxidants play an important role in maintaining the health and wellbeing of fish, by protecting their cells and tissues by scavenging free radicals (Hoseinifar *et al*., 2020). Among the antioxidants analysed in this study, the higher levels of *sod* gene transcripts and enzyme activity in fish fed the COS and FOS diets compared with the control, suggest the fish got the ability to counteract oxidative stress. Increased SOD activity has been observed in FOS fed blunt snout bream (Zhang *et al*., 2014) and MOS fed hybrid grouper (Ren *et al*., 2020), at lower inclusion levels (0.3-2%) in diets. Previous studies have demonstrated that the antioxidant capacity in serum and intestine of fish species depends on the type and dose of the dietary supplement used. One study found that inclusion of 8% insoluble cellulose had no effect on antioxidant activity, but at 16% soluble cellulose and 28% insoluble cellulose, antioxidant capacity

(SOD and CAT levels) was lowered (Deng *et al*., 2021). Another study examining soluble cellulose and mucosal health observed that the higher level of soluble cellulose (>9%) lowered the antioxidant activity of SOD and CAT in the intestine of juvenile largemouth bass (*Micropterus salmoides*) (Liu *et al*., 2022).

5.3.3 Effects of COS on mucosal immunity

In fish, innate immune receptors such as toll-like receptors (*tlrs*) play an important function in recognising bacterial pathogen-associated molecular patterns (PAMPs), which in turn leads to antigen processing and presentation by the adaptive immune system (Rebl *et al*., 2010). In this study, *tlr2* (which recognises bacterial lipopolysaccharides) showed a trend for higher expression (~2- to 5-fold upregulation) in COS and FOS fish compared with the control. Apart from receptors, the complement cascade constitutes an integral part of the teleost innate immune defence (Boshra *et al*., 2006). The significant ($p<0.05$) upregulation of *c3* gene transcript in COS-fed fish compared with the control, indicate greater efficiency of COS as an innate immune stimulator in rainbow trout. Meanwhile, the non-significant transcript level of *c3* gene in FOS fish compared with the control contradicts findings for FOS-treated blunt snout bream (*Megalobrama amblycephala*), where the level showed distinct enhancement (Zhang *et al*., 2014). The *c-type lectin* gene showed distinct elevation in the COS diets; however, only a statistical trend ($p\leq0.1$) could be observed from the transcript expression. Although, the overall gene expression pattern showed upregulation in the COS fed fish, more studies are needed to evaluate the optimal concentration of dietary COS in fish to elucidate its full potential in immunomodulation.

5.4 Implications and impact on sustainability

The research presented in this thesis increases knowledge about alternative microbial and prebiotic ingredients obtained from local municipal and industrial wastes, and from lignocellulosic biomass deriving from inexpensive and renewable substrates. These novel ingredients have advantages over conventional fish meal, soybean meal and rapeseed meal *etc*. in terms of arable land use, dependence on unpredictable weather conditions and fishing restrictions (Tallentire *et al*., 2018). Diets containing

yeast protein concentrate require less agricultural land than *e.g.*, soybean (Tallentire *et al*., 2018) and, compared with animal and plant-based protein sources for food production, microbial protein has 53-100% lower environmental impacts (Järviö *et al*., 2021). As regards environmental burden, various alternative protein meals for salmon, such as yeast-based single-cell protein, have been shown to be more environmentally friendly in terms of six assessment criteria than imported soybean meal (Couture *et al.*, 2019).

Provided that production can scaled up successfully, *N. intermedia* and *Y. lipolytica* can be comparable to fish and soybean meal in fish diets. Therefore, these feed resources have the potential to change the European feed market, which is highly dependent on imported protein-rich feedstuffs such as soybean meal (Tallentire *et al*., 2018). Even small-scale adoption of these novel feeds could contribute to sustainable aquaculture growth and food security (Cottrell *et al*., 2020).

Novel feed ingredients from organic and industrial side-streams or byproducts, for use as a protein source, prebiotic or immunostimulant, are in an early phase of development and the full potential of these sources has not yet been uncovered. In future work, comprehensive analyses covering nutritional benefits, environmental load and economic aspects is required to confirm their potential.

It has been found that modifying culture conditions for microbial biomass (Lapeña *et al*., 2019), using different kinds of substrate or by genetic manipulation, can further improve its nutritional benefits, thus increasing its value as a feed resource in fish diets (Agboola *et al*., 2021). Future studies should investigate this aspect as a matter of urgency.

6. Conclusions and future perspectives

Comparative analysis of the feed trials conducted in this thesis suggested that all three alternative ingredients (*N. intermedia, Y. lipolytica,* and COS, obtained from municipal, industrial and forest wastes, respectively) can be potential alternatives as low-cost, sustainable dietary ingredients for aquafeed. However, their functionality varies with their nature, chemical composition, level of inclusion, downstream processing method and feeding strategies. For instance, *N. intermedia* appeared to be capable of replacing fishmeal as a protein source in fish diets, *Y. lipolytica* was more potent as an immunomodulator and COS can be useful as a prebiotic to modulate gut health and to provide antioxidant capacity. Papers I-IV were the first studies to test these three alternative ingredients in fish feed for rainbow trout, so this thesis provides a wide range of baseline data that can support future research in this area. The main conclusions are as follows

- *Neurospora intermedia* biomass is highly digestible and can be a promising alternative protein source, replacing fishmeal in the diet of rainbow trout. The inclusion levels tested did not compromise fish performance or gut health.

- Dietary *Yarrowia lipolytica* enhanced the mucosal immunity of rainbow trout significantly when included at a level of 5% in the diet. Bioactivity in rainbow trout was more evident for dietary whole *Y. lipolytica* than for autolysed yeast. Also, its protein content being 42%, it can be suitably use to replace auxiliary ingredients like soybean meal rather than fishmeal, which will be of ecologically significant.

- Feeding trials with cello-oligosaccharides did not demonstrate clear immunomodulatory and prebiotic properties, but indicated potential for such properties, so higher inclusion levels in the diet may be needed to achieve maximum functionality of cello-oligosaccharides.

Future perspectives

- The results in this thesis point to a need for further studies to understand the interplay between gut associated microbes and microbial enzymes in presence of bioactive compounds leading to bioactivity.

- To identify bioactive components other than cell wall polysaccharides and to identify the functional mechanisms involved.

- In general, fungal and yeast biomass can capture nutrients from the substrate, so to ensure high nutritional value they could be grown on a variety of substrates and their genetic manipulation could be tested.

- Use of natural resources or biomass can have environmental burdens, so life cycle assessment and economic viability assessment should be performed to ensure that future sustainability goals are met.

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Popular science summary

The sustainable development goals for ‘Blue Transformation’ largely require more efficient, inclusive, resilient and sustainable aquaculture systems. To meet global food demand, aquaculture production is being intensified worldwide, using larger quantities of feed resources for faster and higher growth and production. Fishmeal and fish oil are the principal sources of protein and lipid used in aquaculture diets around the world, particularly for carnivorous fish such as trout and salmonid compared with omnivorous fish such as carp and tilapia. However, global production of fishmeal and fish oil is associated with significant sustainability issues in marine capture fisheries and climate change, which is leading to higher prices and lower availability.

Thus, for sustainable expansion of future aquaculture, alternative feed ingredients are needed. Ingredients based on plant materials, insects, terrestrial animal by-products, microbial biomass and genetically modified organisms are currently being tested to make the aquaculture industry more resilient by lowering its dependency on fishmeal and fish oil. Potentially suitable plant-based feed resources such as soymeal and rapeseed meal are rich in protein, but contain anti-nutritional factors which are detrimental to fish growth and must be removed by expensive feed processing technologies. These plant-based ingredients are also part of the human food and animal feed chain, creating competition for their use in fish feed. Besides, they have an environmental footprint as they are grown on arable land, use soil nutrients and consume precious groundwater.

Efforts are being made to produce alternative nutrient-rich feed ingredients from under-utilised wastes such as municipal sewage, organic industrial by-products and forest by-products. Use of these waste resources

in aquaculture feed could provide a cheap and nutritious alternative to fishmeal, improving sustainability, and would also help 'reduce, reuse and recycle' and become an integral part of the circular bio-economy. Moreover, many waste-based feed ingredients, such as single cell proteins, filamentous fungi, microalgae, yeasts, insect-based protein and ligno-oligosaccharides, contain one or more bioactive compounds (chitin, β-glucan, mannan, lingo-cellulose *etc.*) and are considered to be excellent immune system inducers and gut microbiota modulators in fish.

This thesis evaluated the feed potential for rainbow trout of three classes of dietary ingredients, namely biomass of the filamentous fungus *Neurospora intermedia* obtained as a by-product from the ethanol industry; biomass of *Yarrowia lipolytica* yeast isolated at a municipal sewage treatment plant; and cello-oligosaccharides (lignocellulosic biomass) derived from birch residues.

The potential of *N. intermedia* as a protein source in fish diets was examined by replacing 30% of diet containing fish meal as major protein source. Preconditioned and non-preconditioned fungi diets were tested for digestibility and gut microbiota composition at different day intervals. The *N. intermedia* diets showed high apparent digestibility, comparable to that of the fishmeal-based control diet. There was a gradual shift in overall gut microbiota in fish fed the *N. intermedia* diets, with increased abundance of *Lactococcus* and decreased abundance of *Peptostreptococcus* from day 0 to day 30. No effect of preconditioning on digestibility and gut microbiota was observed in the 30-day feeding trial. These results suggest that duration of feeding and diet are important criteria in shaping gut microbiome.

The effect of including whole and autolysed *Y. lipolytica* at 2% or 5% level in the diet of rainbow trout was examined in a 45-day feeding trial. The results showed positive, but non-significant, impacts of both whole and autolysed yeast on growth performance. Gut microbiota analysis revealed no differences in alpha- and beta-diversity between fish fed the experimental diets. However, log-fold change analysis showed a positive association of *Desulphovibrionaceae* with the whole yeast diets. At 5% inclusion level of whole or autolysed yeast in the diet, *Sphingobactericaceae* showed positive association, while at 2% inclusion level *Rhodobacteraceae* was found to be the dominant microbial taxon. Further, 5% inclusion of whole yeast resulted

in elevated expression of immune-related genes of innate and adaptive pathways in the fish. The higher immunomodulatory effect of whole yeast than autolysed yeast in this respect may have been due to lowering of bioactivity and antigenicity in the autolysed yeast due to sub-optimal heat treatment, while lack of bioactive compounds may have been the reason for the lower immune induction by the 2% whole yeast diet.

The bioactivity of including cello-oligosaccharides (COS) at 0.1%, 0.5% and 1.5% level in the diet of rainbow trout was investigated in a 45-day feeding trial. Inclusion of 0.5-1.5% of COS resulted in higher abundance of *Bacillaceae* and *Lactobacillaceae*, in the gut microbiota. In terms of gene expression analysis, fish fed the COS diets showed only marginal modulation of gut immunity with respect to expression of *c3, c-type lectin* and *tlr2* genes. However, fish fed the COS diets showed higher antioxidant capacity in the gut and serum, indicating higher efficiency in neutralising stress in fish. Analysis of expression levels of cytokine and adaptive immune genes in fish revealed that the COS diets gave no difference compared with the control diet.

In conclusion, the new-generation alternative feed sources tested in this thesis increased bioactivity, either by modulating gut microbiota and/or by immunomodulation, which ultimately resulted in better overall health status of rainbow trout.

Populärvetenskaplig sammanfattning

De hållbara utvecklingsmålen för "Blue Transformation" kräver till stor del effektivare, inkluderande, motståndskraftiga och hållbara vattenbrukssystem. För att möta den globala efterfrågan på livsmedel intensifieras vattenbruksproduktionen över hela världen, genom att använda större mängder foderresurser för snabbare och högre tillväxt och produktion. Fiskmjöl och fiskolja är de viktigaste källorna till protein och lipider som används i vattenbruksdieter runt om i världen, särskilt för köttätande fiskar som öring och laxfisk jämfört med allätande fiskar som karp och tilapia. Den globala produktionen av fiskmjöl och fiskolja är dock förknippad med betydande hållbarhetsfrågor i havsfisket och klimatförändringar, vilket leder till högre priser och lägre tillgänglighet.

För en hållbar expansion av framtida vattenbruk behövs alternativa foderingredienser. Ingredienser baserade på växtmaterial, insekter, landlevande animaliska biprodukter, mikrobiell biomassa och genetiskt modifierade organismer testas för närvarande för att minska beroendet av fiskmjöl och fiskolja och för att göra vattenbruksindustrin mer uthållig. Potentiellt lämpliga växtbaserade foderresurser som sojamjöl och rapsmjöl är rika på protein, men innehåller anti-nutritionella faktorer som är skadliga för fiskens tillväxt och måste avlägsnas med dyra foderbearbetningstekniker. Dessa växtbaserade ingredienser ingår också i foderkedjan för människor och djur, vilket skapar konkurrens om deras användning i fiskfoder. Dessutom har de ett miljömässigt högre fotavtryck eftersom de odlas på åkermark, använder marknäring och konsumerar värdefullt grundvatten.

Ansträngningar görs för att producera alternativa näringsrika foderingredienser från underutnyttjade sidoströmmar från avfallshantering ,

organiska industribiprodukter och skogsbiprodukter. Användning av dessa avfallsresurser i vattenbruksfoder skulle kunna utgöra ett billigt och näringsrikt alternativ till fiskmjöl, förbättra hållbarheten, och skulle också bidra till att "minska, återanvända och återvinna" och bli en integrerad del av den cirkulära bioekonomin. Dessutom innehåller dessa alternativa foderingredienser såsom, filamentösa svampar, mikroalger, jästsvampar, insektsbaserat protein och ligno-oligosackarider, en eller flera bioaktiva föreningar (kitin, β-glukan, mannan, lingo-cellulosa etc.) och anses vara utmärkta immunsysteminducerare och tarmmikrobiotamodulatorer hos fisk.

Denna avhandling utvärderade foderpotentialen hos tre klasser av dietingredienser för regnbågslax, nämligen biomassan av trådsvampen *Neurospora intermedia*, erhållen som en biprodukt från etanolindustrin; biomassa av jästen *Yarrowia lipolytica*, odlad på hydrolysat från organiskt matavfall; och cello-oligosackarider (lignocellulosabiomassa) härrörande från björkrester.

Potentialen för *N. intermedia* som en proteinkälla i fiskdieter undersöktes genom att ersätta 30 % av kosten innehållande fiskmjöl som huvudproteinkälla. Förkonditionerade (värmebehandlade) och icke-konditionerade svampdieter testades med avseende på smältbarhet och sammansättning av tarmmikrobiota efter olika utfordingsintervall. Dieterna visade hög smältbarhet, jämförbar med den fiskmjölsbaserade kontrolldieten. Det skedde en gradvis förändring i den totala tarmmikrobiotan hos fiskar som fick tillskott av *N. intermedia*, med ökad förekomst av laktokocker och minskad förekomst av peptostreptokocker från dag 0 till dag 30. Ingen effekt av förkonditionering på smältbarhet och tarmmikrobiota observerades under 30 dagars utfodringsförsök. Dessa resultat tyder på att utfodringens varaktighet och diet är viktiga kriterier för att forma tarmmikrobiomet.

Effekten av att inkludera hel och autolyserad *Y. lipolytica* motsvarande 2% eller 5% i dieten till regnbågslax undersöktes i ett 45-dagars utfodringsförsök. Resultaten visade positiva, men icke-signifikanta, effekter av både hel och autolyserad jäst på fiskens tillväxt. Analys av tarmmikrobiota avslöjade inga översiktliga skillnader i alfa- och beta-diversitet mellan fiskar som matades med experimentdieterna. Statistisk analys visade dock ett positivt samband mellan andelen

Desulphovibrionaceae och dieten med hel jäst. Dessutom, vid 5 % inklusionsnivå av hel eller autolyserad jäst i kosten visade Sphingobactericaceae en positiv association, medan Rhodobacteraceae var dominerade vid 2 % inklusionsnivå. Vidare resulterade 5% inkludering av hel jäst ett förhöjt uttryck av immunrelaterade gener, både gener relaterade till det medfödda och adaptiva immunförsvaret i fisken. Den högre immunmodulerande effekten av hel jämfört med autolyserad jäst i detta avseende kan ha berott på sänkning av bioaktivitet och antigenicitet i den autolyserade jästen på grund av värmebehandlingen, medan en låg mängd av bioaktiva föreningar kan ha varit orsaken till den lägre immun-induktionen med 2 % hel jästdiet.

Bioaktiviteten av att inkludera cello-oligosackarider (COS) motsvarande 0,1 %, 0,5 % och 1,5 % i dieten av regnbågslax undersöktes i ett 45-dagars utfodringsförsök. Inkludering av 0,5-1,5 % av COS resulterade i högre andel av tarmbakterier, såsom Bacillaceae och Lactobacillaceae. När det gäller analys av genuttryck, visade fiskar som matades med COS-dieter endast marginell modulering av tarmimmunitet med avseende på uttryck av c3, c-type lectin och tlr2-gener. Fiskar som matades med COS-dieterna visade dock högre antioxidativ kapacitet i tarm och serum, vilket tyder på högre effektivitet för att neutralisera stress hos fisk. Analys av uttrycksnivåer av cytokiner och adaptiva immungener i fisk visade att COS-dieterna inte gav någon skillnad jämfört med kontrolldieten.

Sammanfattningsvis ökade den nya generationens alternativa foderkällor som testades i denna avhandling bioaktiviteten, antingen genom att modulera tarmmikrobiota och/eller genom immunmodulering, vilket i slutändan resulterade i bättre övergripande hälsostatus för regnbåge.

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II

frontiers in Marine Science

ORIGINAL RESEARCH
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Dietary Filamentous Fungi and Duration of Feeding Modulates Gut Microbial Composition in Rainbow Trout (*Oncorhynchus mykiss*)

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Changes in gut microbial composition over time in rainbow trout fed differentially processed diets supplemented with the filamentous fungi *Neurospora intermedia* were investigated in a 30-day feeding trial. Fish were fed a reference diet, non-preconditioned diet (NPD), or preconditioned (heat-treated) diet (PD), with the same inclusion level of *N. intermedia* in diets NPD and PD. Gut microbiota were analyzed on day 0, 10, 20, and 30. Gut microbial composition was similar for all diets on day 0, but was significantly different at day 10 and day 20. On day 30, the gut again contained similar communities irrespective of diet. The overall gut microbiota for each diet changed over time. Abundance of *Peptostreptococcus* and *Streptococcus* was higher in the initial days of feeding in fish fed on commercial diet, while a significant increase in lactic acid bacteria (*Lactococcus lactis*) was observed on day 30. Feed processing (preconditioning) did not contribute largely in shaping the gut microbiome. These results indicate that dietary manipulation and duration of feeding should be considered when evaluating gut microbial composition in cultured fish. A minimum 30-day feeding trial is suggested for gut microbiome, host and diet interaction studies.

Keywords: rainbow trout, filamentous fungi, duration of feeding, gut microbiome, fish, *Lactococcus*, amplicon sequencing

INTRODUCTION

Single-cell proteins such as microalgae, bacteria, and fungi are microbial protein sources that represent potential alternatives as fish feed ingredients (Nalage et al., 2016). In particular, filamentous fungi are versatile microorganisms that can grow on a wide range of wastes, industrial by-products, and side-streams. The nutritional value of filamentous fungal biomass derives from its high protein content, fatty acid composition, and presence of other nutrients such as vitamins, minerals, anti-oxidants, and immune stimulant components (Karimi et al., 2019a). Despite these attractive nutritional properties of filamentous fungal biomass, few studies have explored its use as a fish feed ingredient. Using nuclear magnetic resonance (NMR) spectroscopy, Abro et al. (2014) investigated changes in the metabolism of Arctic charr (*Salvelinus alpinus*) fed with filamentous fungal species *Rhizopus oryzae*. In another study, Vidakovic et al. (2016) used intact and extracted baker's yeast (*Saccharomyces cerevisiae*) and *Rhizopus oryzae* as separate diet ingredients and evaluated the effects on digestibility and intestinal barrier function in Arctic charr.

Neurospora intermedia is a food-grade filamentous fungus isolated from traditional fermented food in Indonesia, and is therefore among the filamentous fungi species recognized as safe. Its nutritional properties and cultivation conditions have been extensively explored by our research group (University of Borås) and reported in previous studies (Ferreira et al., 2014, 2015; Gmoser et al., 2018; Karimi et al., 2019b). The high nutritional value of *N. intermedia* and its categorization as a dietary safe microorganism make it an ideal alternative ingredient for fish feed.

The gut microbiota is critical to fish nutrition as it produces several enzymes which help in digestion, transport of nutrients, direct protection from pathogens, and enhanced immunity (Austin, 2006; Merrifield et al., 2010; Camp et al., 2012; de Bruijn et al., 2018). Several studies have found that environmental (abiotic) and host (biotic) factors play important roles in shaping the gut community in fish. Gut microbial composition and diversity are influenced by genetics, sex, weight, age, rearing conditions, diet, and feeding habits (Hovda et al., 2012; Ingerslev et al., 2014a; Li et al., 2016; Ringø et al., 2016; Yan et al., 2016; Sun et al., 2020). High-throughput sequencing has been used previously to explore dietary effects on the gut microbiota of several fish species, such as rainbow trout, Atlantic salmon (*Salmo salar*), Arctic charr, sea bream (*Sparus auratus*), and channel catfish (*Ictalurus punctatus*) (Navarrete et al., 2013; Gajardo et al., 2017; Huyben et al., 2017; Nyman et al., 2017; Wang et al., 2019). Most of these studies have investigated the short- or long-term effect of diet on gut microbiota but, to our knowledge, none has investigated gradual changes in microbial communities over time. Diet can adversely modulate gut microbial composition in fish, leading to inflammation of the distal intestine, as demonstrated for Atlantic salmon fed high levels of soy protein (Gajardo et al., 2017). It has also been shown that Arctic charr fed filamentous fungi (*Rhizopus oryzae*) display higher frequency of diarrhea, despite high apparent digestibility coefficient (Langeland et al., 2016). Knowledge of the interactions between host, gut microbiota, diet, and feeding strategy is important when developing novel diets, in order to ensure better fish health and welfare. The present study sought to extend this knowledge by examining the role of novel filamentous fungi in modulating the intestinal microbiota of rainbow trout over successive 10-day feeding intervals and its efficiency as a fish feed ingredient.

MATERIALS AND METHODS

Fish Husbandry

Juvenile rainbow trout were purchased from Vilstena Fiskodling AB, Fjärdhundra, Sweden, and the experiment was carried out in the Aquatic Facility, Center of Veterinary Medicine and Animal Science, Swedish University of Agricultural Sciences, Uppsala, Sweden. A total of 300 fish (average weight 127.8 ± 19.8 g) were randomly and evenly distributed between 15 oval experimental tanks (200 L) and reared in a 12-h light cycle (08.00–20.00 h). The experimental tanks were equipped with a partial recirculation system and supplied with fresh tap water at 3 L min^{-1}. All fish were judged to be healthy, with no visible signs of injuries detected on skin, gills, or fins. Each experimental tank was connected to a waste feed and feces collection system. Temperature during the whole experiment was 11 ± 1°C and oxygen level was 8 ± 2 mg/L (HQ40D Portable Multi Meter, Hach, Loveland, CO, United States). The fish were acclimatized for 10 days on a commercial diet (Biomar EFICO ENVIRO 920 ADVANCE, 2% of body weight once a day prior to the experiment. The experiment was performed in compliance with laws and regulations on procedures and experiments on live animals in Sweden, which are overseen by the Swedish Board of Agriculture (diary number: 5.8.18-16347/2017).

Production of *Neurospora intermedia* Biomass

Fungal biomass of *N. intermedia* CBS 131.92 (Centraalbureau voor Schimmelcultures, Netherlands) was produced under semi-continuous cultivation condition at the Swedish Center for Resource Recovery, University of Borås. The fungus was cultivated on complex medium containing 30 g/L glucose and 5 g/L yeast extract as the major carbon and nitrogen source, respectively. Trace elements in the form of $(NH_4)_2SO_4$, KH_2PO_4, $CaCl_2.2H_2O$, and $MgSO_4$ $x7H_2O$ in concentrations of 7.5, 3.5, 1.0, and 0.75 g/L were added to the cultivation medium to support filamentous fungi growth, using a 26 L capacity bubble column bioreactor (airlift bioreactor converted to bubble column bioreactor by removing the internal loop tube) (Bioengineering, Switzerland). Cultivation was carried out at 35°C and 1 vvm (volume of air per volume of medium per minute). Cultivation condition parameters and sterilization method were according to Ferreira et al. (2015). To harvest biomass, 75% of the working volume of the reactor (15 L) was harvested twice per day, at 11.00 and 23.00 h. Fresh sterilized cultivation media was added to top up the cultivation broth after harvesting. Harvested broth, containing post-cultivation medium and biomass, was transferred to a cold room and stored at 4°C. After termination of cultivation, biomass was quickly separated from the culture media using a sieve, washed with distilled water, and dried in an air oven at 70°C.

Diets and Feeding

Feed preparation was carried out at the Swedish University of Agricultural Sciences, Uppsala, Sweden. Three experimental diets were prepared, a reference diet (RD), a non-preconditioned diet (NPD), and a preconditioned diet (PD). Diet RD was prepared with fishmeal as the major protein source. Diets NPD and PD were prepared by mixing 30% (by weight) of *N. intermedia* biomass with 70% of diet RD according to Cho (1979). The ingredients were mixed in a kitchen mixer, gelatin dissolved in hot water was added as a binder, and the ingredients were mixed again and pelleted through a meat grinder, using a 3.5 mm die (Nima Maskinteknik AB, Örebro, Sweden). The strings produced were dried in an air oven at 50°C for 12 h and cut into pellets with a twin blade blender (Kneubühler, Luzern, Germany).

Diets PD and NPD were formulated in the same way, but diet PD was preconditioned by heat-processing in a convection

oven (Electrolux Professional, FCE061) at 105°C for 5 min, in order to increase the degree of gelatinization of starch and emulate temperature treatment during extrusion conditions. The prepared feed was stored at −20°C until it was fed to the fish (approximately 2 weeks). Data on feed composition and proximate analysis are presented in **Tables 1**, **2**, respectively. Rainbow trout were fed twice a day throughout the 30-day feeding trial, using automatic belt feeders (Hølland teknologi, Sandnes, Norway). Feed was initially provided in excess (starting with a ration equal to 1.5% of initial body weight) and the ration was adjusted according to the feed waste in the tank.

Sample Collection

Fish were anesthetized with 80 mg/L tricaine methanesulfonate (MS-222, Western Chemical Inc., Ferndale, WA, United States) and weighed at the start and end of the trial, and growth performance was recorded. Sampling for gut microbiota was performed on five fish per treatment at 0, 10, 20, and 30 days of feeding. For this, euthanized fish were aseptically dissected from the ventral side after swabbing with ethanol (70% solution). The hindgut was dissected from the ileocecal valve to 0.5 cm above the anus, and digesta samples and mucosal scrapings were taken. These were snap-frozen in liquid nitrogen and stored at −80°C until DNA extraction.

TABLE 1 | Dietary composition (g kg^{-1} on dry matter basis) of the reference diet (RD), non-preconditioned diet (NPD), and preconditioned (heat-processed) diet (PD).

Ingredients (g kg^{-1})	Diets		
	RD	NPD	PD
Neurospora intermedia	-	298.5	298.5
Fishmeal	420	294	294
Soy protein concentrate	100	70	70
Wheat meal	220	154	154
Fish oil	100	70	70
Rapeseed oil	70	49	49
Carboxymethyl cellulose	10	7	7
Gelatin	60	42	42
Titanium dioxide	05	05	05
Vitamin mineral premix	15	10.5	10.5

TABLE 2 | Proximate composition [g kg^{-1} dry matter (DM)] and energy content (MJ kg^{-1} DM) of the reference diet (RD), non-preconditioned diet (NPD), preconditioned (heat-processed) diet (PD), and *Neurospora intermedia* fungal biomass.

Dietary component	RD	NPD	PD	*N. intermedia*
Dry matter (%)	95.7	95.9	96	–
Crude protein	484	514	516	609
Crude fat	194	171	171	64.5
Neutral detergent fiber	37.7	83.7	87.9	249.9
Ash	85.7	77.8	74.5	83.1
Gross energy	23.4	22.9	22.9	–

Chemical Analysis

Experimental feeds were freeze-dried, milled, and stored at −20°C until analysis. In order to determine the dry matter content, the samples were dried in an oven for 16 h at 103°C and then cooled in a desiccator before weighing. Crude protein content (N × 6.25) (Nordic Committee on Food Analysis, 1976) was determined by the Kjeldahl method, using a 2020 Kjeltec digester and a 2400 Kjeltec Analyser unit (FOSS Analytical A/S, Hilleröd, Denmark). Crude lipid content was analyzed according to the Official Journal of the European Union (2009), using an extraction unit (1047 Hydrolysing Unit and a Soxtec System HT 1043; FOSS Analytical A/S). Neutral detergent fiber (NDF) was measured based on the method described by Chai and Udén (1998) using 100% neutral detergent solution, while amylase and sulphite were used for reduction of starch and protein. Gross energy (GE) content was determined in an isoperibol bomb calorimeter (Parr 6300, Parr Instrument Company, Moline, IL, United States). Dry matter, gross energy, and ash content were analyzed according to standard methods (AOAC, 1995).

Extraction of DNA

Intestinal samples (200 mg) were transferred to sterile cryotubes containing 1 mL InhibitEX buffer and 0.5 g of 0.1 mm silica beads, and homogenized at room temperature in a bead beater (Precellys Evolution, Bertin Technologies) for 2 × 1 min at 6,000 rpm, with a 5 min rest. DNA was extracted using the QIAamp Fast DNA Stool Mini Kit (Qiagen Gmbh, Hilden, Germany) according to the manufacturer's instructions.

Library Preparation and Sequencing

The V4 region of the 16S rRNA gene was amplified from the extracted DNA using the primers 515F (5-GTGCCAGCMGCCGCGGTAA-3) and 805R (5-ACTACHVGGGTATCTAATCC-3). Polymerase chain reactions (PCR) were carried out using Phusion® High-Fidelity PCR Master Mix (New England Biolabs). PCR products were confirmed by gel electrophoresis and were purified with the Qiagen Gel Extraction Kit (Qiagen, Germany) and quantified by Qubit®3.0 Fluorometer (Invitrogen, Thermo Fisher Scientific). Final libraries including barcodes and adaptors were generated with the NEBNext® Ultra™ DNA Library Prep Kit, and the amplicons were then sequenced using Illumina sequencing (NovaSeq 6000) at Novogene (Beijing, China). The BioProject accession number is PRJNA743247.

Bioinformatics Analysis

Paired-end reads were assigned to samples based on their unique barcode. These reads were merged after truncating off the barcode and primer sequence using FLASH (v1.2.7[1]) (Magoč and Salzberg, 2011). Quality filtering on the raw sequence tags was performed using QIIME (v1.7.0[2]) (Caporaso et al., 2010; Bokulich et al., 2013). Sequence analysis by clustering of operational taxonomic units (OTUs) was performed using Uparse software

[1]http://ccb.jhu.edu/software/FLASH/

[2]http://qiime.org/scripts/split_libraries_fastq.html

(Uparse v7.0.1001[3]) (Edgar, 2013). Sequences with ≥ 97% homology were assigned to the same OTUs. Representative sequences for each OTU were screened for further annotation. For each representative sequence, Mothur software was applied to the SSU rRNA data in the SILVA Database[4] for species annotation at each taxonomic rank (Wang et al., 2007; Quast et al., 2012).

Statistical Analysis

A linear mixed effect (LME) model ("nlme" package) was used to test for statistically significant differences between relative proportions of OTUs and diet, sampling day, and diet × day interactions. The LME model results were analyzed using R statistical software version 3.6 (Pinheiro et al., 2014; R Core Team, 2015), considering diet and day as fixed factors and tank as random factor. Data on bacterial OTUs were normalized by log transformation. LME comparison was conducted on OTUs with average abundance > 1%, followed by *post hoc* analysis of emmeans ("emmeans" package) with Tukey adjustment for multiple pairwise comparison. Similarity percentage analysis (SIMPER), analysis of similarity (ANOSIM), principal coordinate analysis (PCoA), principal component analysis (PCA), and Spearman correlation analysis

[3]http://drive5.com/uparse/
[4]http://www.arb-silva.de/

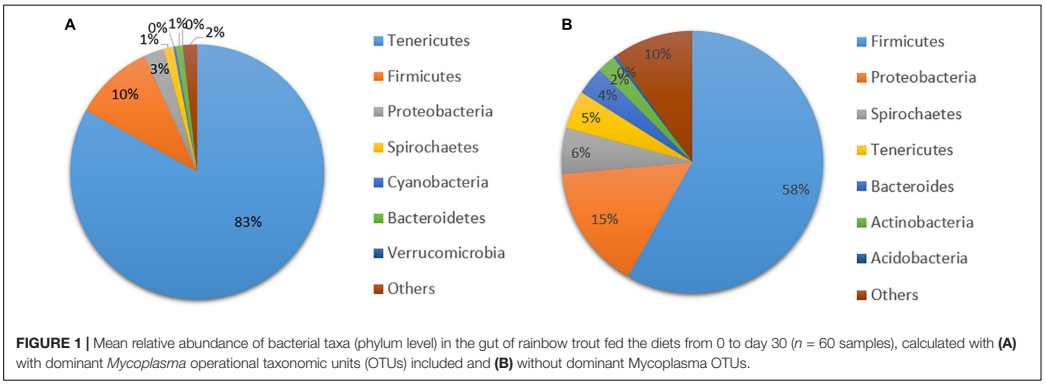

FIGURE 1 | Mean relative abundance of bacterial taxa (phylum level) in the gut of rainbow trout fed the diets from 0 to day 30 (n = 60 samples), calculated with **(A)** with dominant *Mycoplasma* operational taxonomic units (OTUs) included and **(B)** without dominant Mycoplasma OTUs.

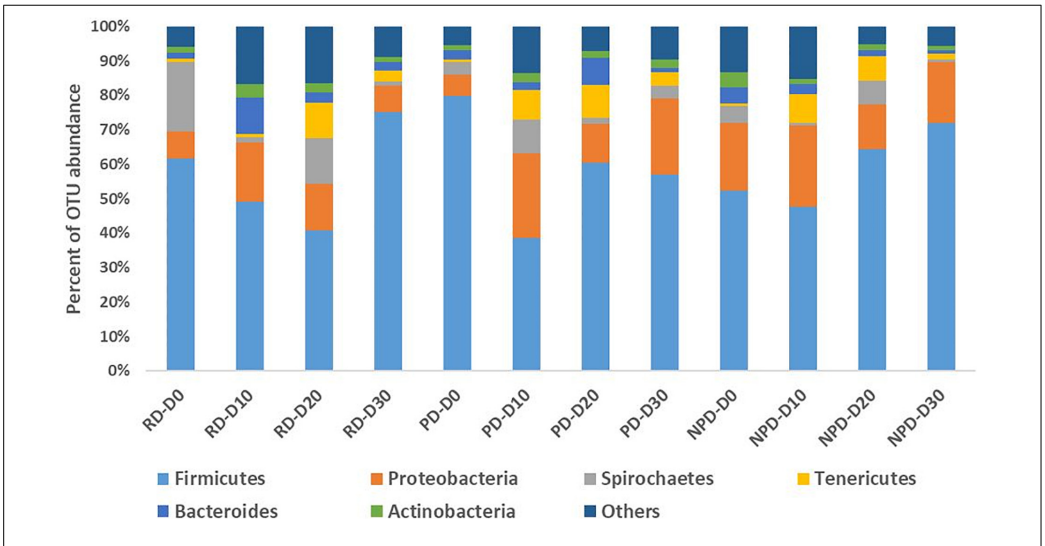

FIGURE 2 | Relative abundance of bacterial taxa in the gut of rainbow trout at different time intervals (after excluding operational taxonomic unit OTU1 and OTU2, belonging to *Mycoplasma*) (n = 5). RD: reference diet, PD: preconditioned (heat-processed) diet, NPD: non-preconditioned diet. D0, D10, D20, and D30 refer to the 10-day time interval. Until day 0, all fish had been fed a commercial diet for 10 days.

were performed using Paleontological Statistics Software version 4.03 (PAST). Two-way ANOSIM was performed to investigate the effect of diet and day interval on beta diversity of gut microbial composition. The ANOSIM and SIMPER analyses were both based on Bray Curtis index, and Bonferroni correction was used to adjust for multiple pairwise comparisons to determine differences in gut microbial composition within and between time intervals for each diet. PCoA based on Bray Curtis and Jaccard dissimilarity was used to assess the overall clustering of samples according to microbial community composition based on diet and time interval.

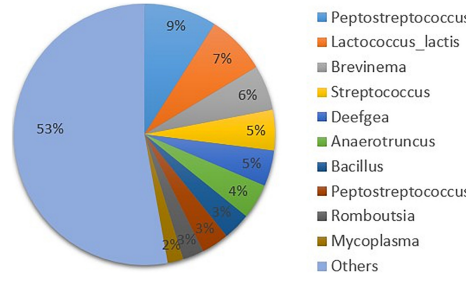

FIGURE 3 | Mean relative abundance of the top 10 bacterial operational taxonomic units (OTUs) in the gut microbiota of rainbow trout from 0 to 30 days on the commercial and experimental diets (n = 60).

RESULTS

Gut Microbial Composition of Rainbow Trout

The overall gut microbial composition after 30 days of feeding showed high dominance of Tenericutes (84%), followed by Firmicutes (10%), and only very low relative abundance of other bacterial phyla. The high dominance of the Tenericutes phylum was due to two dominant *Mycoplasma* OTUs (**Figure 1A**). Assessment of the data did not reveal logical patterns, however, mainly since the two dominant *Mycoplasma* OTUs were not correlated to any of the parameters evaluated. Therefore, in further analyses on microbial composition the two dominant OTUs of *Mycoplasma* were excluded and relative abundance was recalculated, to discern effects on other bacterial taxa. In total, 4.8 million sequence reads of bacteria were obtained. The average number of sequence reads per sample without *Mycoplasma* was 13,244 and the lowest number obtained was 1,398. A total of 5,961 OTUs were obtained after excluding *Mycoplasma* OTUs, and bacterial OTU abundance was then dominated by two phyla, Firmicutes (58%) and Proteobacteria (15%) (**Figure 1B**). The overall trend in bacterial community composition from day 0 to day 30 was that Firmicutes ranged from 38 to 79% and Proteobacteria ranged from 8 to 24% for the different diets (**Figure 2**). Of the top 10 OTUs with abundance > 1% (**Figure 3**), *Peptostreptococcus* (9%), *Lactococcus* (*L. lactis,* 7%), *Brevinema* (6%), *Streptococcus* (5%), *Deefgea* (5%), and *Anaerotruncus* (4%) were the most abundant over the 30-day period.

Shift in Gut Microbial Composition of Rainbow Trout With Diet and Days of Feeding

Principal coordinate analysis was performed to graphically explore the shift in community structure for different diets after different time intervals (**Figures 4A,B**). The percentage variation (PoV) explained for axis 1 and 2 when using Bray Curtis index was 25.5 and 10.3%, respectively. For the analysis based on Jaccard's dissimilarity, PoV explained by axis 1 was 13% and by axis 2 was 6.68%. Differences in gut microbial composition at each day (within interval) were analyzed with one-way ANOSIM. The results confirmed that gut microbial composition for the different diets was similar at day 0 and day 30, but dissimilar at day 10 and day 20 (**Supplementary Table 1**). Pairwise comparison of the treatment groups showed that they were significantly dissimilar within and between 10-day intervals (**Supplementary Table 2**). At day 10, the overall microbial composition of the fish gut with diet PD and NPD was different from that with RD. At day 20 there was a difference in microbial composition between NPD and PD, but they did not differ from RD. Over 10-day intervals, there was a temporal change in gut microbial composition with all diets from day 0 to day 10, from day 10 to day 20, from day 20 to day 30, and from day 10 to day 30 (**Supplementary Table 2**). According to SIMPER analysis, the percentage dissimilarity for the pairwise-compared treatment groups ranged from 64.73 to 82.58%.

Effect of Diet and Days of Feeding on Gut Microbial Composition of Rainbow Trout

Two-way ANOSIM revealed that diet and time had a significant influence in shaping the overall gut microbiota composition of trout (**Supplementary Table 3**). The results from the statistical analysis and interaction plot investigating the effect of treatments within and between 10-day intervals of feeding on the abundance of top six OTUs are shown in **Figure 5**. A more detailed description of these data can be found in **Supplementary Tables 4–6**. Day intervals had significant effects on the abundance of *Peptostreptococcus* and *Streptococcus*. Diet and day had significant effects on *Lactococcus* and *Deefgea*. The abundance of *Anaerotruncus* was significantly affected by diet. An interaction effect was observed only for *Deefgea* and *Anaerotruncus*. At day 30, the abundance of *Streptococcus* was significantly different between diets PD and NPD, while the abundance of *Deefgea* was significantly different for diet RD from NPD and PD (**Supplementary Table 5**). Significant increase in abundance from day 0 to day 30 for all diet namely RD, PD and NPD was only observed for *Lactococcus* (**Supplementary Table 5**). At day 0, *Peptostreptococcus* was the dominant taxon, but by day 30 *Lactococcus* was the most abundant taxon for all diets. The PCA results revealed that the occurrence of *Peptostreptococcus* and *Streptococcus* was positively and negatively correlated, respectively, with that

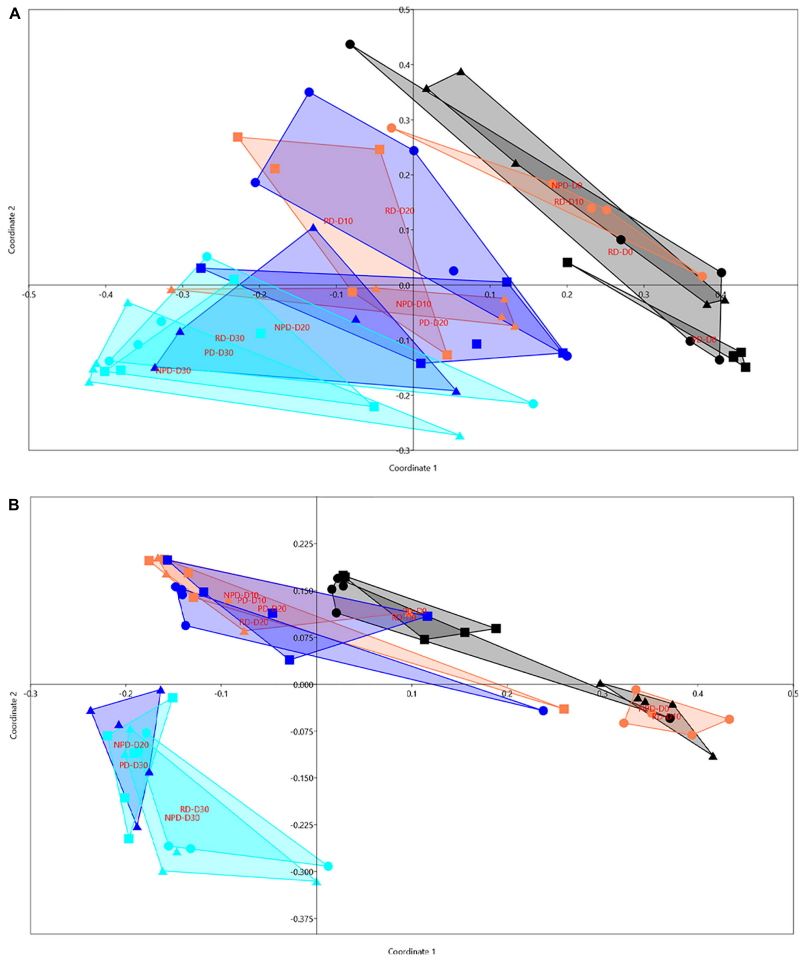

FIGURE 4 | Principal coordinate analysis plot based on **(A)** Bray Curtis index and **(B)** Jaccard dissimilarity showing the shift in gut bacterial community of rainbow trout with diet and time. Reference diet (0), preconditioned diet (□), non-preconditioned diet (Δ). Different colors represent day interval; days 0 (●), 10 (●), 20 (●) and 30 (●). Until day 0, all fish had been fed a commercial diet for 10 days.

of *Lactococcus* (**Figure 6**). These results were confirmed by Spearman correlation analysis (**Supplementary Figure 1**).

Growth Performance

Over the 30-day study period, the fish achieved a mean weight gain of 45.6 ± 3.1%, 44.7 ± 1.5% and 45.1 ± 3.3% for diet RD, NPD, and PD, respectively. These values were not significantly different. All diets were consumed without obvious changes in the intake pattern and zero mortality was recorded during the experimental period.

DISCUSSION

Shift in Overall Gut Microbial Composition With Diet and Time

The PCoA, ANOSIM, and SIMPER results demonstrated that the overall differences seen in gut microbial community were based on type of diet and feeding period. There was a gradual shift in bacterial communities between fish fed the commercial diet (day 0) and those fed the experimental diets (day 10–30) (**Figures 4A,B** and **Supplementary Tables 1**, **2**). Bacterial

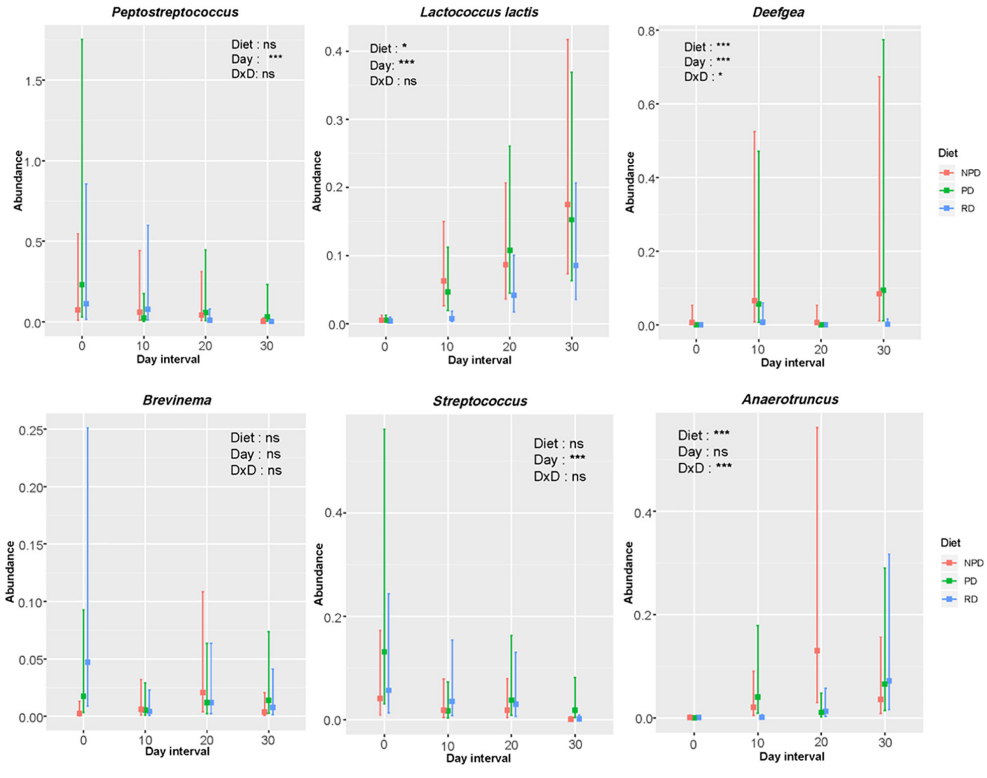

FIGURE 5 | Interaction plot showing relative abundance of the top six bacterial taxa in the gut bacterial community of rainbow trout. RD: reference diet, PD: preconditioned (heat-processed) diet, NPD: non-preconditioned diet. Until day 0, all fish had been fed a commercial diet. The vertical bar in the plot represents confidence interval and overlapping bars means the difference is not significant. Significance: ***$p < 0.001$, ** $p < 0.01$, *$p < 0.05$, and ns $p > 0.05$.

composition was expected to be similar at day 0, since fish in all treatment tanks were fed the same commercial diet during the previous acclimatization period. Within 10 days of experimental diet feeding, microbial composition in the fish gut had changed significantly, indicating that all diets had a significant effect on microbial composition. Previous studies have also reported a change in gut microbiota following a change in diet for salmon, rainbow trout, and brown trout (*Salmo trutta*) after first feeding (Ingerslev et al., 2014b; Michl et al., 2017, 2019). In the studies by Michl and co-workers, trout were fed 0, 50, and 90% plant protein-based diets until 54 days after hatching and then fed a cross-over diet for another 39 days, and a change in microbiome was observed at both 54 and 93 days of feeding showing occurrence of gut microbiota is influenced diet and depend largely on time of sampling. In the present study, diet and 10-day period both had an effect in shaping the gut communities. However, Michl et al. (2017) observed no change in the gut microbiota over time, after a certain point or with longer feeding duration with the same diet, and concluded that microbiota composition depends largely on the actual diet fed at the time of sample collection. In the present study, the gut microbiota differed significantly at day 10 and day 20, but was similar at day 30 irrespective of different treatment diets, suggesting that obtained microbiota at this time point is not influenced by two of the environmental variations in this case heat processing of diet nor *N. intermedia* inclusion. However, temporal change for all diets from day 20–30 was evident. Until day 20, differences in microbial composition can be in order to adapt to the environment due to dietary intervention. Longer periods of study are needed to confirm this.

Little information is available on the effect of thermal processing of feed on the fish gut microbiome. In the presented study, there was no difference in overall microbial composition between the preconditioned (thermal-processed) diet (PD) and the non-preconditioned diet (NPD) at day 30. However, Zhang and Li (2018) observed a decrease in gut microbiota at taxonomic

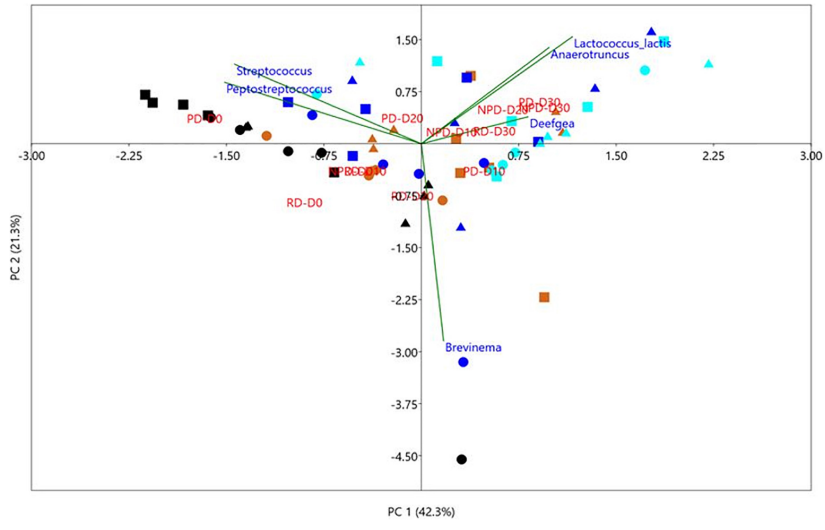

FIGURE 6 | Principal component analysis (PCA) plot showing the correlation of the top six operational taxonomic units (OTUs) in the gut bacterial community of rainbow trout with diet and day interval. Reference diet (0), preconditioned diet (□), non-preconditioned diet (Δ). Different colors represent day interval; days 0 (●), 10 (●), 20 (●), and 30 (●). Until day 0, all fish had been fed a commercial diet for 10 days.

and OTU levels when catfish were fed thermal-processed fish as food (steam, 100°C for 15 min) compared with non-processed food or feed.

Effect of Diet and Duration of Feeding on Core Gut Microbiome

In the present study, high ubiquitous abundance of Tenericutes, dominated by two *Mycoplasma* OTUs, was found which is similar to findings in previous studies on rainbow trout (Lowrey et al., 2015; Lyons et al., 2017a,b; Huyben et al., 2018). According to Holben et al. (2002), *Mycoplasma* can be a natural resident in the gut of both farmed and wild salmon. As the biological function of *Mycoplasma* is not known and very high dominance of the two otus took over the statistical analyses, thus it reduced chance to identify associations among the other microbes possibly associated with the diets.

Data analysis revealed that the next most common phylum in the core gut microbiota after Tenericutes was Firmicutes, folowed by Proteobacteria (**Figure 1B**), as found in other studies on salmonids (Nayak, 2010; Gajardo et al., 2017). One previous study has found that the core gut microbial composition of rainbow trout is resistant to change due to diet type, but that conclusion was reached by comparing data at phylum level (Wong et al., 2013). Another study suggested that there might be differences at lower taxonomic ranks, particularly at species or genus level, rather than at higher taxonomic ranks (Michl et al., 2017). This was the case in the present study, where the abundance of *Lactococcus* (Lactobacillales), *Deefgea* (Neisseriales), and *Anaerotruncus* (Clostridiales) was significantly enriched from day 0 to day 30 and fish on the preconditioned diet (PD) had higher abundance of *Streptococcus* than those on the non-preconditioned diet (NPD). Lower abundance of bacteria of the genera *Deefgea* and *Anaerotruncus* was observed, as also found in the gut microbiota of humans, rainbow trout, and Atlantic salmon isolated through 16s sequencing (Namsolleck et al., 2004; Perez-Fuentes et al., 2018; Ricaud et al., 2018). *Peptostreptococcus* and *Streptococcus* are generally present in high abundance in protein-rich environments, and play an important role in amino acid catabolism and absorption in the gut (Dai et al., 2011; Davila et al., 2013; Neis et al., 2015). The abundance of one taxa can suppress that of another depending on nutrient availability for growth. A shift in microbial composition from *Streptococcus* to *Lactobacillus* has been reported in Atlantic salmon fed fishmeal-free diets or diets with fishmeal replaced with plant protein (Hartviksen et al., 2014). This is comparable to the results in the present study, where *Peptostreptococcus*, the dominant taxon at day 0 (all fish fed the commercial diet) decreased in abundance with time, whereas abundance of *Lactococcus* increased conferring the change due to the substrate exchange.

Diet and Duration of Feeding Promotes Abundance and Dominance of Intestinal *Lactococcus lactis*

Gut bacterial composition in rainbow trout fed plant protein-based diets and Atlantic salmon fed a fishmeal-free diet is

reported to show an increase in abundance of Lactobacillales (Schmidt et al., 2016; Michl et al., 2017). A study using PCR-TTGE-dependent bacterial quantification showed an increase in *Lactobacillus* and *Lactococcus* in Atlantic salmon fed diets in which 30% of the fishmeal was replaced with fermented soy meal (Catalán et al., 2018). Lactic acid bacteria are natural inhabitants of the fish gut and have the ability to adhere and colonize and play a beneficial role in the gut (Seppola et al., 2006; Gatesoupe, 2008). Additionally, growth of *L. lactis* is highly substrate-dependent (Rombouts et al., 2020). It is possible that components in the cell wall of *N. intermedia*, such as beta glucan, chitin, and glycoproteins, act as fermentable substrate for Lactobacillales. Increased Lactobacillales abundance has been observed in Artic charr and rainbow trout fed yeast (Huyben et al., 2017; Nyman et al., 2017). Lactobacillales from fish is known to be slow-growing and the recommended growth period on agar media at low temperatures is up to 4 weeks (Ringø and Gatesoupe, 1998), which is in line with the findings in this study of highest Lactobacillales abundance on day 30. Studies have shown that use of *L. lactis* as a probiotic can enhance weight, immunity, and disease resistance in fish (Sun et al., 2012; Heo et al., 2013; Xia et al., 2018). *Lactobacillus lactis* has also been shown to improve the gut architecture and modulate the intestinal microbial composition in fish (Dawood et al., 2016; Xia et al., 2019; Won et al., 2020).

CONCLUSION

The filamentous fungi *Neurospora intermedia* has a good nutritional profile with high protein content and healthy gut microbiota profile with dominance of lactic acid bacteria. It can be advocated as a protein source to replace fishmeal in the diet of cultured fish, for sustainable feed production and aquaculture. Changes due to environmental interventions, in this case diet and feeding duration were more pronounced for modulating the fish gut microbes at overall and at lower taxonomic levels than feed preprocessing. Preconditioning (steam-processing) of the diet had no effect on shaping the overall microbial gut composition as they were similar on day 30. Diets containing *N. intermedia* promoted abundance of *Lactococcus* compared with the commercial diet. Thus duration of feeding should be taken into account when studying changes in the gut microbial community in rainbow trout following diet manipulation. Based on our findings, a minimum 30-day feeding period is recommended in studies on feed-host interactions. Since, the overall gut microbiota continuously changed until day 30, a future research should investigate the further trends of their occurrence.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: www.ncbi.nlm.nih.gov/bioproject, PRJNA743247.

ETHICS STATEMENT

The animal study was reviewed and approved by Swedish Board of Agriculture (diary number: 5.8.18-16347/2017).

AUTHOR CONTRIBUTIONS

TL and AK conceived the study and experimental design. AS and SK carried out the experimental trial, participated in sampling of the fish material, and wrote the first draft of the manuscript. AS, SK, AV, and ML were performed the feed optimization and feed production. AS and JD were responsible for the DNA analysis and performed the data analysis. TL, AV, and JD participated in editing the final manuscript. SK, JF, and MT were involved in fungus production. All authors contributed to manuscript revision, and read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.728569/full#supplementary-material

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This thesis investigated the dietary potential of three alternate feed sources obtained from industrial by-products, municipal side-streams and forest residues in rainbow trout. The results showed that these feed sources modulated the gut microbiota, improved mucosal immunity and contributed to overall health in rainbow trout. Thus, they can be considered as functional feed for the rainbow trout. However, their functionality varies with their nature, chemical composition, levels of inclusion, downstream processing method and feeding strategies.

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