Evaluation of microbial ingredients as alternative, sustainable protein sources for fish meal replacement in rainbow trout feed

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Abstract

The growing demand for sustainable aquaculture has intensified the need for alternative protein sources that can consistently replace fish meal in salmonid diets. Many current alternatives, particularly plant-based proteins, compete directly with human food resources. Microbial ingredients present a promising solution because they can valorize low-value substrates while providing high-quality nutrients. Although bacterial meals and single-cell fungi have been studied extensively, the use of filamentous fungi as feed ingredients for salmonids remains comparatively underexplored.

This licentiate thesis investigates the potential of several filamentous fungi, cultivated on industrial and forestry by-products, as feed ingredients for rainbow trout. Key nutritional and functional aspects were evaluated, including digestibility, effects on growth performance and gut health, and physical pellet quality. In Paper 1, four filamentous fungi namely, *Aspergillus oryzae*, *Rhizopus oligosporus*, *Rhizopus delemar*, and *Paecilomyces variotii*, were assessed for their chemical composition, amino acid indices, and digestibility. Their influence on pellet quality was also examined. Among these, *P. variotii* showed the most favourable characteristics and was selected for further evaluation in a growth trial using graded inclusion levels.

In Paper 2, a nine-week growth trial was conducted with diets containing 0% (control), 5%, 10%, 20%, and 30% *P. variotii*. Growth performance, feed utilization, gut health parameters, and technical feed quality were assessed. The results showed no reduction in growth performance compared with the control up to a 20% inclusion level, while performance declined at 30%. Notably, villus length was significantly higher in fish fed the 30% inclusion diet.

In conclusion, *P. variotii* was found to be the most suitable microbial ingredient among the tested ingredients and hence was chosen for further analysis. *P. variotii* up to 20% inclusion levels did not have any effects on the growth. Further studies with longer feeding periods are required to further judge the efficacy of *P. variotii* as a suitable microbial ingredient for rainbow trout.

Keywords: Rainbow trout, Filamentous fungi, growth performance, gut health, technical pellet quality

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Dedication

To my family who have been supporting me throughout my journey. To friends and colleagues.

Contents

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List of Publications

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

I Ashwath Gaudhaman, Sajjad Karimi, Torbjörn Lundh, Margareth Øverland, Mohammad J. Taherzadeh, Markus Langeland, Kartik Baruah and Aleksandar Vidakovic (2025). Fungal Protein from Non-Food Bioresources in Diets for Rainbow Trout (*Oncorhynchus mykiss*). Fishes MDPI Volume 10, Page 149 (Published)

II Ashwath Gaudhaman, Aleksandar Vidakovic, Niklas Warwas, Darragh Doyle, Byron Morales-Lange, Margareth Øverland, Torbjörn Lundh, Kartik Baruah (2025). *Paecilomyces variotii* mycoprotein in diets for juvenile rainbow trout (*Oncorhynchus mykiss*): Effects on growth performance, intestinal health, and technical feed quality. (Manuscript)

The contributions of AG to the papers in this thesis were as follows:

I Conceptualization with assistance, software, formal analysis, investigation, data curation, writing—original draft preparation, writing—review and editing, visualization.

II Conceptualization, formal analysis, investigation, methodology, visualization, writing – original draft, writing – review and editing.

Abbreviations

FAO	Food and Agriculture Organization.
ANFs	Anti-nutritional factors
PEK	*Paecilomyces variotii*
AO	*Aspergillus oryzae*
RO	*Rhizopus oligosporus*
RD	Rhizopus delemar
DM	Dry matter
PDI	Pellet durability index
PAS	Periodic acid–Schiff
HE	Haematoxylin and Eosin
SGR	Specific growth rate
FBW	Final body weight
IBW	Initial body weight
FCR	Feed conversion ratio
ADC	Apparent digestibility coefficient
CS	Chemical score
EAAI	Essential amino acid index
ANOVA	Analysis of variance
AIC	Akaike's information criterion
WSI	Water stability index
AD	Apparent digestibility
AD_{DM}	Apparent digestibility of dry matter
AD_{CP}	Apparent digestibility of crude protein
AD_{CF}	Apparent digestibility of crude fat
AD_{AA}	Apparent digestibility of amino acids
AD_{Ash}	Apparent digestibility of ash
WG	Weight gain
SCC	Short circuit current
TER	Transepithelial resistance
TEP	Transepithelial potential
NSP	Non-soluble polysaccharides
HSI	Hepatosomatic index
VSI	Viscerosomatic index

1. Background

1.1 The Need for Sustainable Aquafeed Ingredients

1.1.1. Global Aquaculture: An Overview

Aquaculture has emerged as the fastest-growing food production sector, contributing significantly to global food security and economic development [2]. Global aquaculture production reached a new record of 130.9 million tonnes in 2022 (Figure 1), valued at USD 313 billion and comprising 94.4 million tonnes of aquatic animals and 36.5 million tonnes of algae (FAO 2024). As capture fisheries reach their maximum sustainable yield, aquaculture now supplies more than half of the world's fish for human consumption [2]. This increase in aquaculture production occurred mainly in finfish aquaculture (58.1 %), followed by crustaceans (24.6 %) and molluscs (15.6 %). The United Nations Food and Agriculture Organization (FAO) has predicted that global aquaculture production will increase further by two-fold by 2050 [3].

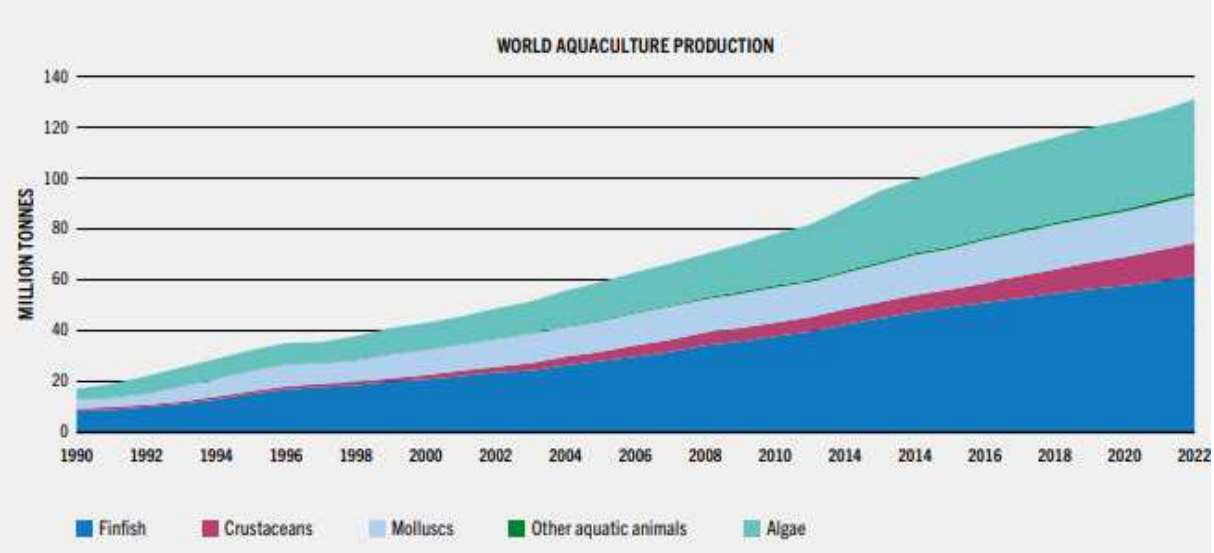

Figure 1. Global aquaculture production (Source: [2])

Aquaculture development rate varies significantly both across and within different geographical regions. A few major producers dominate the production of key groups of farmed species. Asia has accounted for approximately 91.4% of the world's aquaculture production, followed by Latin America and the Caribbean (3.3 %), Europe (2.7 %), Africa (1.9 %), North America (0.5 %) and Oceania (0.2 %) [2]. China stands out as a major producer of farmed food fish, consistently producing more than the rest of the world since 1991. Other leading producers include Indonesia, India, Vietnam, Bangladesh, the Philippines, the Republic of Korea, Norway, Egypt and Chile [2]. In the Nordic region, salmonids, such as Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), and Arctic charr (*Salvelinus alpinus*), are some of the most produced finfish. Globally, the salmonid species accounts for about 6.9% of the total finfish production, with volumes of 4,243,000 tones [2]. Rainbow trout is the most valuable species farmed in the EU in 2023, accounting for 17.7% of all aquaculture production value (Figure 2).

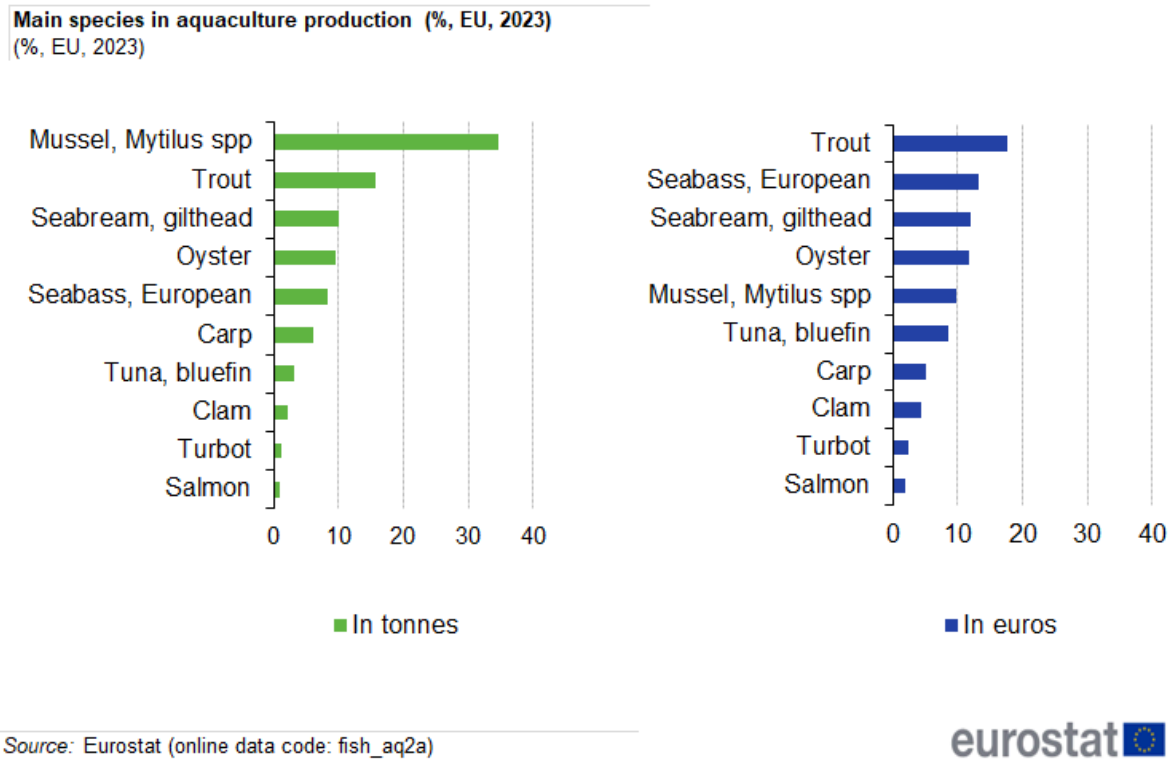

Figure 2: Eurostat production of main aquaculture species in terms of weight and value (Source: [4]).

1.1.2. Swedish Aquaculture: An Overview

Rainbow trout makes up 87% of Sweden's food fish production (Figure 3, Sweden-statistics 2024), making it an important commercial fish in Sweden. The second most farmed is Arctic char, followed by Atlantic salmon (Jordbruksverket – Vattenbruk 2024). However, despite Sweden's favorable natural conditions, including a long coastline and numerous lakes, aquaculture production has stagnated in recent years. This stagnation is primarily attributed to complex and slow permitting processes, stringent environmental regulations associated with nutrients or waste disposals from farming activities, and the absence of designated aquaculture zones in municipal planning [5]. The strategic plan highlights the importance of innovation, environmental sustainability, and economic viability, with particular emphasis on fish feed [5].

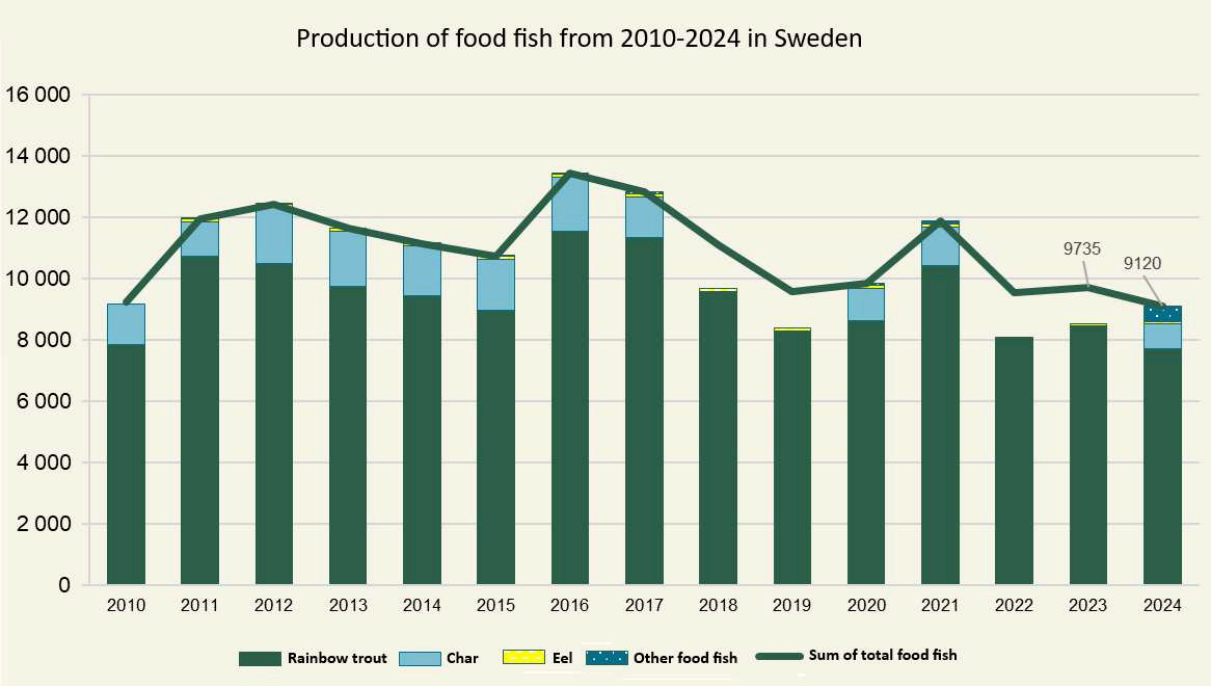

Figure 3: Production of food fish from 2010 to 2024 in Sweden (Translated from figure source: [6]

1.1.3. The need for novel feed ingredients

The above scenario has intensified the demand for high-quality, nutritionally balanced fish feed to ensure optimal growth performance, health, and reduced environmental footprint across a wide range of cultured species in Sweden [7]. In rainbow trout farming, feed constitutes the largest share of production expenses, often making up 40 to 70% of the total costs [8]. As such, the profitability of trout culture largely depends on factors related to feed, such as its nutritional value, the feed formulation pattern followed, and the feeding strategy employed, including ration size and frequency of feeding [9]. In particular, the Swedish aquaculture industry is under increasing pressure to identify sustainable and cost-effective alternatives to conventional feed ingredients, such as fish meal and soybean meal, which are limited in supply and/or subject to price volatility. Fishmeal and fish oil are derived mostly from wild-caught forage fish such as Peruvian anchovies and sardines, even though the use of by-products is increasing [10]**.** Their use contributes to overfishing, which disrupts marine food webs and threatens species that rely on these fish [11-13]. Some species that are under acute threat in the Baltic Sea include cod (*Gadus morhua*) and herring (*Clupea harengus*). Fishmeal supply can also not be predictable, making it a highly volatile commodity [14]. Addressing this challenge is critical to support the continued growth of aquaculture while minimizing its environmental footprint.

As aquaculture moves toward greater efficiency, it must also transform its approach to feed ingredients, not only their sources, but in the mindset guiding their use. This requires balancing multiple considerations, which include nutritional value with environmental impact, short-term growth with long-term sustainability, and economic feasibility with circularity potential. Fish meal is considered the major protein source in rainbow trout feed due to its favorable amino acid profile, high digestibility, and high palatability, all of which support optimal growth performance and health. Soy protein concentrate is one of the most significant plant-based protein sources in rainbow trout nutrition, but it comes with both notable advantages and limitations. The advantages include that they have good amino acid composition, high protein content and do not depend on finite fishery resources

[15]. The disadvantages include that they are produced from soybeans, which can be directly consumed by humans and require cultivable land to grow on.

1.2. Limitations of Current Fishmeal Alternatives

To reduce reliance on the unsustainable fish meal in rainbow trout feed, a variety of alternative protein sources, such as soy protein concentrate, wheat gluten, insect meal, and poultry by-product meal, have commonly been used, each offering distinct nutritional profiles, availability, and sustainability considerations. These alternative ingredients are commonly used in varying inclusion levels in combination with fish meal. The factors that contribute to the inclusion levels include the nutritional value, chemical composition, digestibility and palatability, presence/absence of anti-nutritional factors, cost-effectiveness, EU approval of the ingredient, and its impact on fish growth performance, health, and final product quality [16, 17].

Soybean and cereal cultivation require large amounts of arable land, water, and agrochemicals, which could lead to deforestation, loss of biodiversity, and water pollution. Furthermore, dependence on imported commodities like soy and fishmeal exposes the aquafeed manufacturers to international trade disruptions and geopolitical risks. On the other hand, animal by-products such as blood meal, despite being rich in nutrients, often face skepticism related to upscaling and availability, food safety, especially in Europe, where concerns over diseases such as bovine spongiform encephalopathy (e.g., mad cow disease) have significantly influenced feed regulations [18]. Traceability and variability in the quality of these ingredients can also pose challenges, as they increase the risk of contamination with substances, such as heavy metals and pathogens [19].

In salmonids, several plant-based protein sources have issues with poor palatability [7, 20]. Another main problem is the presence of anti-nutritional factors (ANFs) such as protease inhibitors, phytates, saponins, which can impair digestibility, reduce palatability and/or interfere with intermediary metabolism [21] in carnivorous fish. Another example is that plant protein sources have a high fiber content, such as cellulose, hemi-cellulose and lignin, which limits their nutritional value, making them less suitable for salmonids. Furthermore (as previously mentioned), products derived from plant-based grains also require intensive use of resources, as they utilize arable land and freshwater (among others), making them less sustainable for feed production [22]. Especially considering that, plant derivatives from soybeans are widely used in terrestrial livestock and human food sectors, leading to price volatility and supply competition. This also poses an ethical dilemma, as food that could be directly consumed by humans is used as feed, even though global hunger persists.

1.3. Microbial Ingredients as an Alternative Novel Protein Source

Microbial ingredients have gained attention as sustainable alternatives to conventional protein sources in aquafeeds for salmonids such as rainbow trout. These ingredients include proteins derived from bacteria, yeasts, and filamentous fungi, as well as microalgae. Their appeal lies in high protein content, favorable amino acid profiles, and the potential for production on non-arable land using industrial or agricultural waste streams. Examples include *Methylobacterium extorquens*, *Candida utilis*, *Saccharomyces cerevisiae*, and *Paecilomyces variotii*, many of which have shown promising results in terms of digestibility and growth performance in salmonids, mainly Atlantic salmon [23, 24]. Moreover, certain microbial ingredients offer functional benefits such as immune modulation or gut health support. For instance, Morales-Lange et al. [25] has shown that the addition of *Debaryomyces*

hansenii in the diets of Atlantic salmon has improved the acute stress response in terms of cortisol and immunoglobulin modulations. Despite these advantages, challenges remain regarding cost, scalability, and regulatory approval for widespread inclusion in commercial trout feeds.

Microbial ingredients have a unique advantage, along with plant-based sources, in that they skip trophic levels. This improves efficiency in the utilization of resources [26]. In addition, microbial ingredients are able to use inorganic nitrogen and carbon sources to produce biomass [7]. The cultivation of microbial ingredients also offers a significant advantage over other alternatives. For instance, they can be cultivated year-round and don't depend on seasons [27]. Additionally, they do not compete with arable land that can be used to produce crops, which in turn can be used for human consumption [28].

1.3.1. Single-cell Microbial Ingredients in Aquafeeds

Several microbial ingredients have been evaluated as feed ingredients in salmonids, such as rainbow trout. Bacterial meal has been shown to have a similar amino acid composition as fish meal [29]. Yeasts are also well known for their ability to convert low-quality by-products into high-quality feed. Conversion of lignocellulosic biomass into yeast biomass has been explained methodically in Øverland and Skrede [30]. Species such as *Saccharomyces cerevisiae* and *Candida utilis* have been studied for their nutritional value and functional benefits, including improved gut health and immune modulation on Atlantic salmon [15, 24]. Cell wall components of hydrolyzed *Debaryomyces hansenii* yeast have been shown to have acute stress-mitigating properties and immunomodulation properties when exposed to acute hypoxia [25].

1.3.2. Filamentous (Multicellular) Fungi in Aquafeeds

Among microbial ingredients, multicellular fungi occupy a unique intersection of biological efficiency and ecological relevance. These organisms are capable of converting low-value substrates, such as agricultural or industrial by-products, into high-quality biomass rich in protein, essential amino acids, and functional compounds [30]. Compared with fish meal, fungi are generally more sustainable and resource-efficient [24, 31]. Their rapid growth rates, relatively low resource requirements, and adaptability to controlled fermentation systems make them an efficient and scalable alternative to conventional feed ingredients [24, 31]. Many fungal species also possess a high crude protein content; for instance, certain yeasts contain around 50% crude protein, which is comparable to the 34–42% typically found in soybeans [15, 32, 33]. Furthermore, their potential to reduce the environmental impact of aquaculture by lowering reliance on fishmeal and soy-based proteins highlights their ecological significance in the development of sustainable salmonid feeds. Moreover, a diverse array of fungal species is available, enabling selection based on specific feed requirements. In addition to their nutritional value, fungal components can exert beneficial physiological effects, such as enhancing immune responses [34].

The nutritional composition of the filamentous fungi varies depending on the species, culture conditions and substrate used [35]. The crude protein content of the biomass can be as high as 66.8% with a decent essential amino acid index. Protein is the major energy source for salmonids. It is also essential for supporting growth, tissue repair, and overall metabolic function. In addition to protein, filamentous fungi may provide beneficial lipids, B-complex vitamins, minerals, and bioactive polysaccharides like β-glucans [36-39]. These compounds not only contribute to nutritional value but

may also support immune function and gut health. However, the presence of indigestible components such as chitin and chitosan in fungal cell walls can affect nutrient availability, necessitating appropriate processing [40]. The cell wall is also an important component of fungal biomass and serves as an important functional feed ingredient [41, 42].

The structural complexity of fungal mycelia, primarily composed of chitin and β-glucans, can influence nutrient digestibility in monogastric animals, such as fish. These polysaccharides, while forming an essential part of the fungal cell wall, are generally resistant to endogenous digestive enzymes in teleosts [43]. This suggests that high inclusion levels of intact mycelia in feed formulations may hinder nutrient accessibility by encapsulating intracellular nutrients or increasing digesta viscosity. However, the extent of this effect depends on the fungal species, degree of processing (e.g., autolysis, enzymatic disruption, or mechanical shearing), and inclusion level [40, 44]. Processing methods that partially degrade or disrupt the mycelial matrix can improve digestibility by increasing nutrient bioavailability while preserving valuable bioactive compounds [40, 44]. Therefore, understanding the physicochemical characteristics of the fungal biomass used and tailoring processing strategies are critical for optimizing the nutritional performance of mycelium-based feed ingredients. Additionally, the fungal ingredients cultivated using different waste streams or resource substrates are likely to exhibit variations in their nutritional composition, including differences in protein content, amino acid balance, lipid profile, mineral concentration, and presence of bioactive compounds [27]. These compositional differences can, in turn, influence the growth performance, feed efficiency, immune response, gut health, and overall welfare of farmed fish in diverse ways. Comparative studies on farmed fish are warranted to determine how these differences influence growth performance, nutrient utilization, immune function, gut microbiota, and overall health. Such studies should aim to elucidate the species-specific responses to fungal-based feed ingredients and identify the optimal inclusion levels that maximize benefits without compromising feed efficiency or product quality. Such comparative studies will contribute to evidence-based formulation strategies for aquaculture feeds, supporting both nutritional adequacy and environmental sustainability.

1.4. Rainbow Trout Nutrition and Suitability of Fungal Protein

Rainbow trout are among the most extensively studied farmed fish species in the context of feed, feeding, and nutrition. As naturally carnivorous fish with a high trophic level (ranging from 3 to 4.5), they typically consume small fish, insects, and crustaceans [9]. Consequently, they rely heavily on dietary protein to meet their energy requirements and have a limited capacity to utilize carbohydrates, particularly complex ones [45]. Therefore, the carbohydrate content in their diet should be kept minimal. However, carbohydrates cannot be entirely excluded, as plant-based protein sources inherently contain some carbohydrates, and carbohydrates also play a functional role in feed manufacturing, especially during extrusion.

According to NRC (2011), the dietary requirements for rainbow trout are approximately 38% crude protein and 4200 kcal of energy/kg of diet. Due to their carnivorous nature and relatively simple digestive systems, rainbow trout are especially sensitive to the quality of dietary ingredients. Their evolutionary adaptation to high-protein, animal-based diets means that variations in ingredient digestibility, nutrient composition, and the presence of antinutritional factors can significantly affect their growth performance, health status, and feed conversion efficiency [16, 46]. Given these nutritional and physiological considerations, the selection of alternative protein sources for rainbow trout must ensure high digestibility, favorable amino acid profiles, and minimal antinutritional factors. In this context, multicellular fungi have emerged as promising candidates [44, 47]. There are reports

suggested that certain fungal species can provide high-quality protein with balanced amino acid composition, low carbohydrate content, and favorable digestibility in salmonids [24, 48]. Moreover, their cell walls are typically more digestible than those of microalgae or some plant-based ingredients, and they often lack the antinutritional factors commonly found in terrestrial plants [49]. Thus, multicellular fungi have the potential to serve as sustainable feed ingredients that align well with the dietary requirements and digestive capabilities of rainbow trout.

Microbial ingredients such as single-cell proteins or bacterial meals can affect the physical quality of rainbow trout pellets [50]. Bacterial meal replacement affects the extrusion process and produces pellets with enhanced water stability, likely due to the formation of cross-linked protein networks during processing [51]. On the other hand, some microbial-derived ingredients (e.g., single-cell proteins) have been associated with relatively unstable fecal and pellet residues, which may affect waste properties in aquaculture systems [52]. The net effect on pellet quality, therefore, depends strongly on the type of microbial ingredient, inclusion rate, and processing conditions.

Given the growing interest in incorporating carbon-neutral feed ingredients into salmonid diets, there is a pressing need to better understand their nutritional value, digestibility, and overall impact on fish performance and environmental sustainability. Therefore, a more comprehensive and comparative study should be undertaken to evaluate the efficacy of these novel feed ingredients in trout. Such research would provide critical insights into their potential as sustainable alternatives to conventional feed components and support the development of environmentally responsible aquaculture practices.

2. Hypothesis

Dietary replacement of fish meal and/or soy protein concentrate with alternative, sustainable protein sources from microbial ingredients [e.g., *Paecilomyces variotii* (or PEKILO®; PEK), *Aspergillus oryzae* (AO), *Rhizopus oligosporus* (RO), and *Rhizopus delemar* (RD)] will support pellet quality, growth performance in rainbow trout, as well as beneficially modulating the intestinal health of the fish.

3. Objectives

3.1. Main Objective

To identify and characterize alternative and/or sustainable microbial protein sources for novel aquafeeds, aiming to support pellet quality, growth performance and intestinal health of rainbow trout.

3.2. Specific Objectives

• To determine the digestibility of various filamentous fungal ingredients in rainbow trout and identify the most suitable candidate for a growth performance experiment.

• To establish the optimal inclusion levels of microbial ingredients in novel feeds for rainbow trout.

• To evaluate the health effects of selected microbial ingredients at different inclusion levels on the intestinal health of rainbow trout.

• To integrate the overall impact of microbial ingredients on pellet quality, digestibility, fish growth performance and intestinal health of rainbow trout, contributing with the optimal inclusion levels for the different ingredients in diets for salmonids.

4. Materials and Methods

4.1. Experimental overview

The overview of the workflow for the different papers is presented in Figure 3. The thesis comprises two parts. The first experiment (Paper 1) focused on determining the digestibility of the four microbial ingredients in a 70:30 ratio. The second experiment (Paper 2) was a growth trial conducted to examine the effects of the microbial ingredient PEK at different inclusion levels on the growth performance and gut health parameters of rainbow trout.

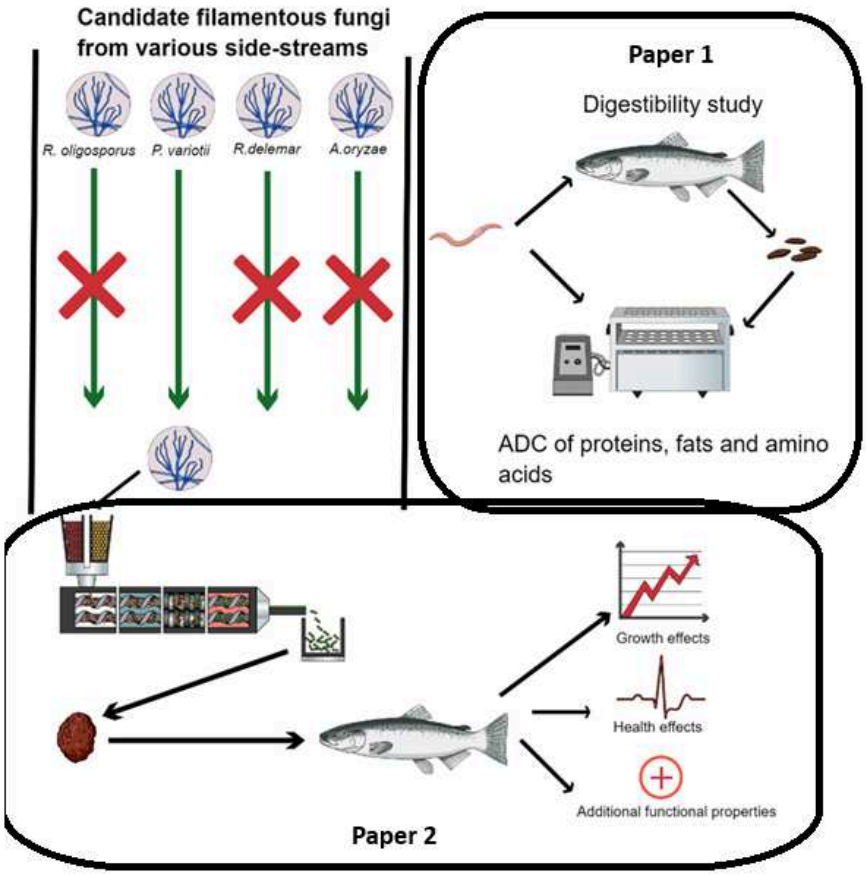

Figure 4: Overview of the workflow for the two experiments. The first experiment (Paper 1) is focused on determining the digestibility of the test microbial ingredients in a 70:30 ratio. The second experiment (Paper 2) was a growth trial conducted to examine the effects of a selected ingredient (PEK) at different inclusion levels on the growth and health status of rainbow trout. ADC: Apparent digestibility coefficient.

4.2. Experimental setup

The overall experimental setup of the two experiments is presented in Table 1.

Table 1: Comparison of the experimental setup for the two experiments.

	Paper 1	**Paper 2**
Fish species	Rainbow trout	Rainbow trout
Average Initial body weight	61.8g	43g
Period	39 days	77 days
Number of diets	5 diets	5 diets
Replicates; Total tanks	3; 15	3; 15
Yeast species	*P. variotii* *A. oryzae* *R. oligosporus* *R. delemar*	*P. variotii*
Aim	Digestibility	Growth trial
Fish meal replacement	30%	0%, 5%, 10%, 20% and 30%

4.3. Microbial ingredient production

A total of four microbial ingredients were tested. These test ingredients included *P. variotii* strain KCL-24 (or PEKILO®; PEK), *A. oryzae* CBS 819.72 (AO), *R. oligosporus* CBS 112.07 (RO), and *R. delemar* CBS 145940 (RD). PEK was produced in an aerobic continuous fermenter using French sugar beet vinasse, a byproduct of bio-ethanol production, as the substrate. The vinasse was diluted to provide 20 g/L of utilizable carbon sources, mainly glycerol and residual sugars, and supplemented with $(NH_4)_2SO_4$ (5 kg), KCl (150 g), $MgSO_4 \cdot 7H_2O$ (150 g), and Vogel's trace elements. The medium was continuously fed to the fermenter at a dilution rate of approximately 0.3 h^{-1} at 37 °C. Biomass was harvested continuously at the same rate using a Larox filter press (Lappeenranta, Finland), then ground and dried in a fluid bed dryer at ~65 °C to a dry matter content of ~94%.

RD and AO were cultivated on thin stillage supplied by Lantmännen Agroetanol (Norrköping, Sweden) using submerged fermentation in a demo-scale reactor (1000 L working capacity, Process- & Industriteknik AB, Kristianstad, Sweden). The inoculum (20 L) was prepared from spores via two-step cultivation: initially in 1 L shake flasks, followed by a 26 L airlift bioreactor. Thin stillage was diluted 1:4 with tap water, heat-sterilized at 121 °C, and fermented at 35 °C for 72 h at pH 4.7 ± 0.3, without additional nutrient supplementation. The resulting fungal biomass was harvested, dewatered, pressed, dried at 60 °C, and milled before use.

RO was produced on dried whole stillage from Lantmännen Agroetanol, adjusted to 50% moisture, in a solid-state fermentation demo plant (Millow AB, Västra Frölunda, Sweden) at 30 °C for 24 h, without added nutrients. After fermentation, the biomass was dried at 60 °C and milled. The proximate composition of the ingredients is presented in Table 2.

Table 2: Proximate composition (g/kg DM), energy (MJ/kg DM), and amino acid (g/kg DM) content of the microbial ingredients.

	PEK	**AO**	**RO**	**RD**
Dry matter %	94.2	92.8	95.6	95.9
Ash content	93	76	16	84
Crude protein	668	441	487	493
Gross energy	21.3	22.3	21.7	21.5
Crude fat	41	125	34	61
Essential amino acids				
Arginine	26.8	15.4	16.7	16.2
Histidine	9.7	8.9	9.1	10.7
Isoleucine	19.0	16.4	16.6	16
Leucine	33.7	28.4	30.1	26.5
Lysine	29.1	20.9	13.4	23.1
Methionine	9.8	6.6	7.6	6.6
Phenylalanine	20.9	16.2	18.4	16.3
Threonine	18.8	16.4	15	17.1
Valine	21.5	20.1	19.9	19.2
Non-essential amino acids				
Alanine	26.7	20.4	20.1	19.4
Aspartic acid	40.1	28.1	29.7	32.8
Cysteine + Cystine	5.5	4.3	9.4	5.8
Glutamic acid	82.3	54.9	101	58.3
Glycine	25.3	17.7	16.6	16.6
Proline	25.3	27.2	31.2	33.5

4.4. Formulation of diets

4.4.1. Paper 1

The experiment included five diets: one reference diet and four test diets. Each test diet consisted of 70% reference diet and 30% of the respective test ingredient, as previously described [53]. The composition of the feeds is provided in Table 3. All diets were produced using a laboratory twin-screw extruder (Ketse 20/40, Anton Paar TorqueTec [Brabender] GmbH, Duisburg, Germany) with five heating zones and a 2 mm die head at the Feed Technology Laboratory of the Swedish University of Agricultural Sciences, Uppsala.

Table 3: Feed composition of the control and the test diets. All units are expressed in g/kg on a DM basis. Titanium dioxide was used as an inert marker for digestibility calculations.

	Diets [1]				
	Control	**PEK**	**AO**	**RO**	**RD**
Fish meal [2]	400	280	280	280	280
Soy protein concentrate [3]	100	70	70	70	70
Wheat gluten [4]	110	77	77	77	77
Wheat meal [5]	200	140	140	140	140
Pot starch [6]	10	7	7	7	7
Fish oil [7]	159	111	111	111	111
Vitamin mineral premix [8]	10	7	7	7	7
PEK		300			
AO			300		
RO				300	
RD					300
Monocalcium phosphate [9]	10	7	7	7	7
Titanium dioxide	1	1	1	1	1
Total	1000	1000	1000	1000	1000

[1] PEK—PEKILO®, AO—*A. oryzae*, RO—*R. oligosporus*, and RD—*R. delemar*. [2] Group 1 fish meal, Pelagia, Bergen, Norway. [3] HP310, Hamlet Protein A/S, Horsens, Denmark. [4] Repal GL21, Lantmännen Reppe AB, Lidköping, Sweden. [5] Wheatmeal standard, Axfood AB, Sweden. [6] Potatismjöl, Axfood AB, Sweden. [7] Fish oil herring, AB Salmonfarm Oy, Kasnäs, Finland. [8] Per kg of premix: Vit A 2,266,667 IU/kg, Vit D3 1,000,000 IU/kg, menadione 6667 mg/kg, thiamine 6000 mg/kg, riboflavin 8667 mg/kg, pantothenic acid 26,667 mg/kg, pyridoxine 5667 mg/kg, Vit B12 20,000 µg/kg, nicotinic acid 50,000 mg/kg, folic acid 3333 mg/kg, biotin 263,667 µg/kg, Vit C 90,000 mg/kg, inositol 165,000 mg/kg, zinc 25,000 mg/kg, iodine 1067 mg/kg, copper 1318 mg/kg, manganese 1640 mg/kg, citric acid 180 mg/k, BHT 536 mg/kg, BHA 256 mg/kg. [9] MCP—Monocalcium phosphate, Aako, Leusden, the Netherlands.

4.4.2. Paper 2

In this study, *P. variotii* (PEK) was examined in a comprehensive manner to determine its potential growth-promoting and health-beneficial effects. To this end, five iso-nitrogenous and iso-energetic diets were formulated, comprising one commercial-like control and four experimental diets with increasing inclusion levels of *P. variotii*. The control diet reflected standard industry practice, whereas the experimental diets contained 5% (D5), 10% (D10), 20% (D20), and 30% (D30) inclusion levels of PEK. The test ingredient was incorporated by proportionally replacing fish meal and soy protein concentrate. Yttrium oxide (Y_2O_3) was included as an inert marker to assess nutrient digestibility. All diets were produced at the Feed Technology Laboratory, SLU, Uppsala, Sweden, using a Ketse 20/40 twin-screw extruder (Brabender GmbH & Co. KG, Duisburg, Germany) equipped with a 2 mm die head. Pellets were dried in a vertical drying oven (Elvärmedetaljer, Skurup, Sweden) and subsequently coated with oil using a mini GVC-10 vacuum coater (Amandus Kahl GmbH & Co. KG, Reinbek, Germany). The detailed composition of the diets is provided in Table 4.

Table 4: Feed composition of the control and experimental diets with novel ingredients. All units are expressed on g/kg basis. D_0 – control (0% PEKILO® inclusion), D_5 – 5% PEKILO® inclusion, D_{10} – 10% PEKILO® inclusion, D_{20} – 20% PEKILO® inclusion and D_{30} - 30% PEKILO® inclusion

	Diets				
Ingredients	**D_0**	**D_5**	**D_{10}**	**D_{20}**	**D_{30}**
Fish meal[1]	390	365	340	290	240
Soy protein concentrate[2]	148	123	100	50	0
Wheat gluten[3]	60	60	60	60	60
Wheat meal[4]	120	120	120	120	120
Potato starch[5]	93	93	88	85.5	80
Fish oil[6]	79	79	80	81	84
Rapeseed oil[7]	80	80	80	80	80
Vitamin mineral premix[8]	10	10	10	10	10
Pekilo	0	50	100	200	300
Lysine sulfate[9]	0	0	1	2	3.7
Choline chloride[10]	5	5	5	5	5
DL-methionine[11]	0	0.3	0.7	1.6	2.2
Monocalcium phosphate[12]	15	15	15	15	15
Yttrium oxide	0.1	0.1	0.1	0.1	0.1

[1] Group 1 fish meal, Pelagia, Bergen, Norway. [2]HP310, Hamlet Protein A/S, Horsens, Denmark. [3]Repal GL21, Lantmännen Reppe AB, Lidköping, Sweden. [4]Wheatmeal standard, Axfood AB, Sweden. [5]Potatismjöl, Axfood AB, Sweden. [6]Fish oil herring, AB Salmonfarm Oy, Kasnäs, Finland. [7] Rapeseed oil feed grade, Avena Nordic Grain Oy, Helsinki. [8]Per kg of premix: Vit A 2,266,667 I Ukg^{-1}, Vit D3 1,000,000 I Ukg^{-1}, menadione 6667 mg kg^{-1}, thiamine 6,000 mg kg^{-1}, riboflavin 8,667 mg kg^{-1}, pantothenic acid 26,667 mg kg^{-1}, pyridoxine 5,667 mg kg^{-1}, Vit B12 20,000 µg kg^{-1}, nicotinic acid 50,000 mg kg^{-1}, folic acid 3,333 mg kg^{-1}, biotin 263,667 µgkg^{-1}, Vit C 90,000 mg kg^{-1}, inositol 165,000 mg kg^{-1}, zinc 25,000 mg kg^{-1}, iodine 1067 mg kg^{-1}, copper 1318 mg kg^{-1}, manganese 1640 mg kg^{-1}, citric acid 180 mg kg^{-1}, BHT 536 mg kg^{-1}, BHA 256 mg kg^{-1}. [9] L-Lysine Monohydrochloride (L-Lysine HCl), MEIHUA, Langfang, Hebei, China [10] MIAVIT GmbH, Essen, Germany, [11] MetAMINO® DL-methionine, Evonik Nutrition & Care GmbH, Essen, Germany.[12]MCP—Monocalcium phosphate, Aako, Leusden, the Netherlands. [13] Yttrium oxide (Y_2O_3) Sigma-Aldrich Sweden AB, Stockholm, Sweden.

4.5. Experimental Fish and Facilities

The experiment described in Paper 1 was conducted at Vattenbrukscentrum AB in Kälarne, Sweden. The digestibility trial (Paper 1) was conducted for 39 days and involved a total of 296 rainbow trout juveniles, all previously reared and acclimatized to the culture conditions of the same facility. Groups of 20 fish, with an average weight of 61.8 ± 15.3 g, were randomly distributed across 15 tanks. Each tank had a volume of 340 L with flow-through water at 10 L/min and a mean temperature of 11.6 ± 1.9 °C. The water, sourced from Lake Ansjön, was pre-filtered using a drum filter, and its dissolved oxygen content was approximately 8.5 mg/L. Each of the five experimental diets (i.e., control, AO, RO, RD, or PEK) was fed to triplicate groups. The diets were fed manually once daily in the morning (10:00 h), starting at 2% of their body weight. Feeding levels were subsequently adjusted to satiation based on uneaten feed. Fish were monitored every other day for abnormal swimming behavior and mortality throughout the trial.

Feces were collected by stripping once during week 4 and twice per week during weeks 5 and 6, totalling five collections. Prior to stripping, fish were sedated in the tanks using 40 mg/L of tricaine methanesulfonate (MS-222). Individual fish were then removed and anesthetized with 80 mg/L MS-222) (Western Chemical Inc., Ferndale, WA, USA). Excess water on the fish was carefully removed to prevent contamination of the samples. Feces were collected by gently squeezing the posterior intestine, following the method of Austreng [54]. After sampling, fish were returned to tanks containing fresh water. Fecal samples from each tank were pooled and stored at −20 °C for subsequent analysis. At the end of the final stripping, fish were euthanized using a lethal dose of 240 mg/L MS-222.

The experiment described in Paper 2 was conducted at the Aquatic Facility of the Department of Applied Animal Science and Welfare (SLU, Uppsala, Sweden). Juvenile rainbow trout with an average initial weight of 43 ± 10 g were obtained from Vilstena Fiskodling. A total of 450 fish were randomly distributed into 15 experimental tanks, with 30 individuals per tank, ensuring comparable initial mean weights among tanks to minimize variation. Each tank had a capacity of 200 L. Rearing conditions were maintained at a 12:12 h light–dark cycle, a temperature of 12.9 ± 0.3 °C, and a dissolved oxygen level of 8.8 ± 0.3 mg/L. Fish were fed twice daily, at 11:00 and 15:00 h, using automated belt feeders (Hølland Teknologi, Sandnes, Norway) operating for one hour per feeding session. Feces from the previous day were collected daily before the start of feeding and weighed periodically. Uneaten feed was separated from feces, and both were stored for later analysis. Feeding rations were adjusted based on the amount of uneaten feed remaining from the previous day. Fish were batch-weighed at the beginning and end of the trial.

4.6. Fish sampling and analysis

4.6.1. Proximate analysis

The proximate composition analyses of the feed and fecal samples in all two experiments were carried out following the same standard procedure. Feed samples were milled into fine particles, while fecal samples were freeze-dried prior to analysis. Dry matter was determined by drying subsamples at 103 °C for 16 h, cooling them in a desiccator for 2 h, and then weighing. For ash determination, the dried samples were incinerated in a muffle furnace at 550 °C for 3 h, followed by cooling in a desiccator and weighing. Total nitrogen content was analyzed using the Kjeldahl method with a 20 digester, an 8400 Kjeltec analyzer unit, and an 8460 sampler unit (Foss, Hillerød, Denmark). Crude protein content was calculated as N × 6.25 [54]. Crude fat was determined using the Soxhlet method with a Soxhlet

extraction system (1047 Hydrolyzing Unit, Soxtec System HT 1043, FOSS Analytical A/S). Titanium dioxide (TiO_2) was used as an inert marker and analyzed following the method described by Short et al. [55], using spectrophotometry at 410 nm (UV 1800 Shimadzu, Kyoto, Japan).

4.6.2. Pellet Quality Analysis

Finished oil-coated pellets were used to evaluate pellet quality. Thirty pellets were randomly selected and arranged in ascending order based on their length. The middle fifteen pellets were chosen for analysis, and their length, width, and hardness were measured on the same samples. Length and width were determined using electronic calipers, while hardness was measured with a hand-held hardness tester (Herkules M, Amandus Kahl GmbH & Co. KG, Reinbek, Germany). Hardness was expressed as the force (kg) required to break a pellet. Pellet durability was assessed using a New Holmen Portable Pellet Tester (NHP100, Holmen Feed, Norfolk, UK) following the procedure described by Wolska et al. [56]. Briefly, pellets were pre-sieved, and 100 g of the sample were subjected to 70 mbar air pressure for 120 s while being continuously sieved. The samples were then weighed to calculate the pellet durability index (PDI). Each diet was tested in triplicate, and the apparatus was cleaned between runs to remove residual oil and debris. Sinking velocity was determined by randomly selecting 35 pellets and recording the time taken for each to sink 1 m in still tap water at 20 °C. Water stability was analyzed following the procedure of Baeverfjord et al. [57] with modifications to the incubation times. The pellets were incubated for 30, 90, and 180 min.

4.6.3. Histology

Distal intestine segments approximately 3–5 mm long were fixed in 10% formalin for 48 h. Following fixation, the samples were dehydrated through a graded ethanol series (50–100%) according to Purushothaman et al. [58]. The dehydrated tissues were then embedded in paraffin, sectioned at 5 µm thickness, and stained with Periodic acid–Schiff (PAS) and Haematoxylin and Eosin (HE) following the procedure described by Hellman et al. [59]. Stained sections were examined using a Nikon microscope equipped with a Nikon DXM1200 camera and Nikon ACT-1 software (Version 2.70). Villi length was quantified using ImageJ 1.54g as outlined by Rocha et al. [60], while goblet cell area measurements were performed using the same software following the method of Raskovic et al. [61].

4.6.4. Ussing chamber analysis

To assess the influence of *P. variotii* on intestinal permeability and ion transport, *ex vivo* measurements were performed using the Ussing chamber technique as described by Warwas et al. [62]. Briefly, the intestine was divided into proximal (extending from the final pyloric caecum to the ileorectal valve) and distal (from the ileorectal valve to the anus) segments. Each section was opened longitudinally and mounted in modified Ussing chambers (Grass & Sweetana [63]. Both sides of the chamber were filled with 4 mL of chilled Ringer's solution, continuously aerated, and maintained at 12°C using a water-cooled jacket.

4.7 Calculations for growth indices, digestibility, pellet quality and amino acid scores

The growth performance indices were calculated using the following formula:

Total dry feed intake (g) = Total feed given (on dry matter basis) (g) − Uneaten feed (on dry matter basis) (g)

Weight gain (g) = Final weight (g) − Initial weight (g)

Corrected weight gain (g) = Weight gain (g) − (Number of fishes dead × average initial weight of the fishes (g))

Corrected weight gain (%) = Corrected weight gain (g)/Total initial weight gain (g) × 100

Specific growth rate (SGR% day^{-1}) = $\frac{ln\ (FBW) - ln\ (IBW)}{Experimental\ period\ (days)}$ x 100

Corrected FCR = $\frac{Total\ dry\ feed\ intake\ (g)}{Corrected\ weight\ gain\ (g)}$

The dietary apparent digestibility (AD%) of dry matter, protein, and fat was calculated using the following formulae described by Cho and Slinger [50] and modified by Bureau et al. [61]:

$ADC_{nutrient/energy} = \left[1 - \frac{(Marker_{feed} x Nutrient_{feces})}{(Marker_{feces} x Nutrient_{feed})}\right]$

$ADC_{dry\ matter} = \left[1 - (Marker_{feed}/Marker_{feces})\right]$

where

$Marker_{feed}$ = Marker content as % of dry matter of the feed;

$Marker_{faeces}$ = Marker content as % of dry matter of the feces;

$Nutrient_{feed}$ = Nutrient content as % of dry matter of the feed;

$Nutrient_{faeces}$ = Nutrient content as % of dry matter of the feces.

The ADCs of the test ingredients were calculated using the following equation adopted from [61]:

$AD_{ingredient} = AD_{testfeed} + (AD_{testfeed} - AD_{ref.feed}) \times \left[\frac{0.7\ x\ Nutrient_{ref.}}{0.3\ x\ Nutrient_{ingredient}}\right]$

Where:

$Nutrient_{ref.}$ = nutrient content as % of reference diet (as is);

$Nutrient_{ingredient}$ = nutrient content as % of test ingredient (as is).

Water stability = (Final dry sample/Initial dry sample) × 100

Pellet expansion = ((Pellet width − Die diameter)/Die diameter) × 100

The protein quality of each ingredient was assessed using chemical scores (CS) as described by Mitchell et al. [64] and later modified by Veldkamp [65]. The chemical score (Figure 4) evaluates the amino acid composition of each ingredient relative to fish meal and to one another. Since the CS examines each amino acid separately, it does not provide an overall measure of amino acid adequacy. To address this, the essential amino acid index (EAAI) was calculated to obtain a single composite score reflecting the

balance of all essential amino acids according to the nutritional requirements of rainbow trout. The EAAI was determined using the formula described by Oser BL [66]based on the integrated amino acid composition of the ingredients (Figure 5).

$$\textbf{EAAI} = \sqrt[n]{\frac{aa1}{AA1} \times \frac{aa2}{AA2} \times \frac{aa3}{AA3} \ldots\ldots\ldots\ldots \frac{aan}{AAn}}$$

This index allowed for comparison and identification of ingredients with the most favorable amino acid profiles.

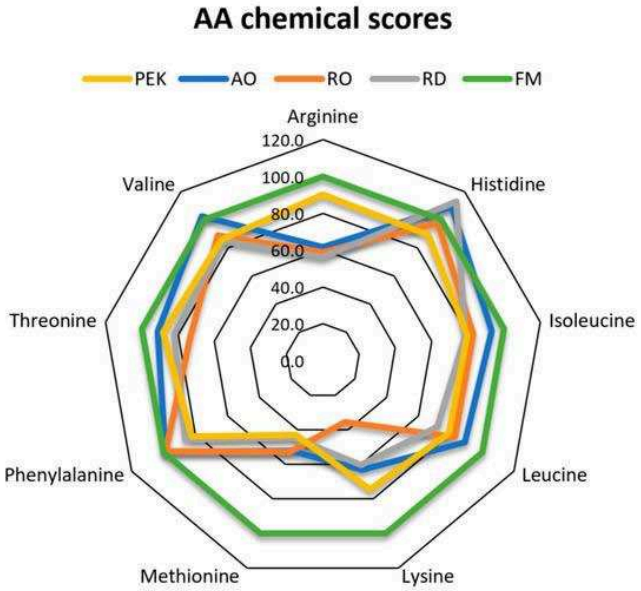

Figure 5. Radar chart showing the amino acid chemical scores (CSs) for the different filamentous fungi compared to fish meal (FM). The value for fish meal was obtained from the 'Feed Ingredient Composition Database (FICD) v10.0 **https://app.iaffd.com/ficd** (accessed on 25 October 2024) [1]. The results are presented as percentages of amino acid content of crude protein in the test ingredient compared with the amino acid content of crude protein in the fish meal. A score of 100 would indicate that the amino acid content in the ingredient matches the amino acid content in fish meal. The scale is in multiples of 20, ranging from 0 to 120.

4.8 Statistical analyses

In paper 1, statistical analyses were performed using GraphPad Prism version 10.1.0 (316). Group means were compared using one-way analysis of variance (ANOVA), and differences were considered significant at $p < 0.05$. When the ANOVA indicated significant effects, pairwise comparisons were conducted using Tukey's test under the same significance threshold.

In paper 2, all statistical analyses were conducted in RStudio 2024.04.2 Build 764. Assumptions of normality and homogeneity of variance were evaluated prior to ANOVA; normality was assessed using the Shapiro–Wilk test and Q–Q plots, while homogeneity of variance was examined using Levene's test. One-way ANOVA was then performed with dietary inclusion level as the fixed factor, and effects were considered significant at $p < 0.05$. When significant differences were detected, the EMMEANS procedure was used for pairwise comparisons among dietary groups. Linear and quadratic regression analyses were also conducted using dietary inclusion level as a continuous predictor, and their significance is reported as p-linear and p-quadratic. To determine the best-fitting model, Akaike's

Information Criterion (AIC) was calculated for both linear and polynomial regressions, with the model exhibiting the lowest AIC selected for interpretation.

5. Results

5.1. Proximate composition of the ingredients

The analyzed proximate composition of the microbial ingredients used in Papers 1 and 2 is shown in Table 2. On a dry-matter basis, the crude protein content of the filamentous fungi ranged from 44.1% in AO to 66.8% in PEK. Crude fat content varied from 3.4% in RO to 12.5% in AO, while the gross energy content ranged between 21.3 MJ/kg DM (PEK) and 22.3 MJ/kg DM (AO).

5.2. Physical pellet quality

In Paper 1, the pellet diameter, expansion ratio and water stability index (WSI, %) values at 30, 60, and 120 min are presented in Table 5. Among the diets, the control showed the highest expansion ratio, while the RD diet exhibited the lowest. After 30 min, the control diet displayed the highest WSI, and RO the lowest, with no significant differences among the remaining diets. By 60 min, the differences became more pronounced. The control and PEK diets showed the highest WSI values, followed by AO, RD, and RO. After 120 min, the variation among diets further increased, with the control diet maintaining the highest WSI (82.9%) and the RO diet the lowest (54.9%).

Table 5. Pellet width, expansion, and water stability index (WSI %) of the experimental diets. PEK—PEKILO®, AO—*A. oryzae*, RO—*R. oligosporus*, and RD—*R. delemar*.

Pellet Quality Parameter	Control	PEK	AO	RO	RD	Pooled SEM [1]	*p*-Value [2]
Pellet width (mm)	2.8^{a}	2.6^{b}	2.3^{c}	2.3^{c}	2.1^{d}	0.05	<0.0001
Expansion (%)	37.8^{a}	29.0^{b}	13.4^{c}	17.0^{c}	4.0^{d}	2.42	<0.0001
WSI (%) 30 min	92.1^{a}	89.6^{ab}	84.9^{ab}	82.6^{b}	86.1^{ab}	2.73	<0.0001
WSI (%) 60 min	89.0^{a}	86.1^{ab}	79.2^{bc}	72.6^{c}	77.7^{c}	1.84	0.0030
WSI (%) 120 min	82.9^{a}	73.2^{b}	63.5^{c}	54.9^{d}	65.3^{bc}	4.63	0.0296

[1] Pooled standard error of the mean. [2] Significance of one-way ANOVA. Values in the same row with different superscripts indicate significant differences as determined by Tukey's multiple comparison test ($p \leq 0.05$).

In Paper 2, the pellet quality parameters are summarized in Table 6. Pellet width and expansion appeared unaffected by the varying inclusion levels of the test ingredients. Pellet durability, however, showed a significant quadratic decline with increasing inclusion levels of *P. variotii* ($p_{value} < 0.0001$; $p_{linear} = 0.0019$; $p_{quadratic} < 0.0001$). No significant linear or quadratic trends were observed for sinking velocity, although the control diet displayed a significantly higher sinking velocity than the D_5 and D_{10} diets ($p_{value} = 0.0022$; $p_{linear} = 0.806$; $p_{quadratic} = 0.09718$). Water stability after 30 minutes showed a probable linear increase with higher inclusion levels of *P. variotii* ($p_{value} = 0.0292$; $p_{linear} = 0.0005$; $p_{quadratic} = 0.0028$). At 90 and 180 minutes, a significant quadratic relationship was observed instead

(p_{value} = 0.0289; p_{linear} = 0.0045; $p_{quadratic}$ = 0.0092). Across all time points, the D_{30} diet exhibited the greatest water stability.

Table 6: Physical pellet quality parameters for the different diets used in paper 2. D_0 – control (0% *P. variotii* inclusion), D_5 – 5% *P. variotii* inclusion, D_{10} – 10% *P. variotii* inclusion, D_{20} – 20% *P. variotii* inclusion and D_{30} - 30% *P. variotii* inclusion.

Physical pellet quality	**D_0**	**D_5**	**D_{10}**	**D_{20}**	**D_{30}**	**SEM**	**p-value**	**p-linear**	**p-quadratic**	**AIC**
Pellet width (mm)	2.53	2.64	2.48	2.63	2.53	0.0478	0.0756	0.9494	0.8249	L
Expansion (%)	26.5	32.13	24.0	31.63	26.5	0.2708	0.0756	0.9494	0.8249	L
Durability (%)	90.6^{a}	91.1^{a}	89.2^{a}	90.5^{a}	71.5^{b}	0.4626	<0.0001	0.0019	<0.0001	Q
Sinking velocity (m sec^{-1})	0.072^{a}	0.060^{b}	0.068^{ab}	0.067^{ab}	0.068^{ab}	0.0021	0.002	0.806	0.097	Q
Water stabiltiy index (%)	**D_0**	**D_5**	**D_{10}**	**D_{20}**	**D_{30}**	**SEM**	**p-value**	**p-linear**	**p-quadratic**	**AIC**
30 min	88^{b}	89^{ab}	90^{ab}	91^{ab}	92^{a}	0.0076	0.0292	0.0005	0.0028	L
90 min	83^{b}	85^{b}	85^{b}	86^{ab}	89^{a}	0.0075	0.003	0.0002	0.0006	Q
180 min	78^{ab}	76^{b}	80^{ab}	80^{ab}	83^{a}	0.0121	0.0289	0.0045	0.0092	Q

Different superscript letters within a row indicate statistically significant differences between means ($p < 0.05$). *P*-value refers to the ANOVA, whereas p-linear refers to the linear regression model and p-quadratic refers to the quadratic regression model. AIC stands for Akaike information criterion which is used to determine which model describes the dataset best. An AIC value 'Q' indicates that the quadratic model describes the response variable better than the linear model whereas 'L' indicates the vice-versa.

5.3. Apparent digestibility

5.3.1. Apparent digestibility (%) of the experiment ingredients

The AD (%) of the ingredients from Paper 1 are presented in Table 7. The apparent digestibility of dry matter (AD_{DM}) ranged from 23.6% in RD to 59.3% in PEK. Among the tested ingredients, PEK exhibited a significantly higher AD_{DM} than AO, RD, and RO. The apparent digestibility of crude protein (AD_{CP}) varied between 44.9% and 86.5%, with PEK showing the highest value. RO displayed a significantly higher AD_{CP} than AO, whereas RD had a significantly lower AD_{CP} compared with AO, RO, and PEK. The apparent digestibility of crude fat (AD_{CF}) ranged from 83.8% (AO) to 90.5% (RD), with no significant differences among the ingredients. The apparent digestibility of essential amino acids (AD_{AA}) is presented in Table 7, along with pooled standard errors of the mean. Methionine digestibility ranged from 55.3% in RD to 91.5% in PEK, while lysine digestibility ranged from 56.7% in RO to 93.8% in PEK.

Table 7. Apparent digestibility (AD%) of the experimental ingredients from Paper 1. PEK—PEKILO®, AO—*A. oryzae*, RD—*R. delemar,* RO—*R. oligosporus,* and Control—control diet.

	PEK	AO	RO	RD	Pooled SEM[1]	p-value[2]
Dry matter	59.3^{a}	31.3^{b}	24.1^{b}	23.6^{b}	6.346	0.0003
Crude protein	86.5^{a}	56.5^{c}	71.0^{b}	44.9^{d}	2.041	<0.0001
Crude fat	87.4	83.8	88.5	90.5	4.967	<0.0001
Essential amino acids						
Arginine	93.9^{a}	78.3^{b}	72.4^{bc}	68.9^{c}	2.482	<0.0001
Histidine	92.0^{a}	66.6^{b}	54.2^{c}	54.3^{c}	3.323	<0.0001
Isoleucine	90.5^{a}	67.6^{b}	52.2^{c}	50.7^{c}	3.501	<0.0001
Leucine	91.1^{a}	69.3^{b}	57.4^{c}	54.8^{c}	3.458	<0.0001
Lysine	93.8^{a}	74.1^{b}	56.7^{c}	58.1^{c}	3.413	<0.0001
Methionine	91.5^{a}	69.7^{b}	61.8^{bc}	55.3^{c}	3.902	<0.0001
Threonine	88.4^{a}	58.0^{b}	52.0^{b}	46.5^{b}	5.004	0.0001
Valine	89.7^{a}	67.7^{b}	50.6^{c}	49.5^{c}	3.938	<0.0001
Total AA	90.5^{a}	68.0^{b}	57.2^{b}	56.3^{b}	3.692	<0.0001

[1] Pooled standard error of the mean. [2] Significance of one-way ANOVA. Values within the same row that bear different superscript letters are significantly different according to Tukey's multiple comparison test ($p \leq 0.05$).

5.3.2. Apparent digestibility of the experimental diets

In Paper 1, the apparent digestibility (AD) values of diets are presented in Table 8. The apparent digestibility of dry matter (AD_{DM}) in the control diet was 83.3%. All test diets showed significantly lower AD_{DM} values than the control, ranging from 65.1% in RD to 76.0% in PEK. Diets containing PEK had significantly higher dry matter digestibility compared with those containing AO, RD, or RO, while no significant differences were observed among the AO-, RD-, and RO-based diets. The apparent digestibility of crude protein (AD_{CP}) ranged from 76.8% in RD to 91.2% in the control diet. The ADCP of all test diets was significantly lower than that of the control, except for the PEK diet, which did not differ significantly. The apparent digestibility of crude fat (AD_{CF}) ranged from 90.5% (RD) to 95.3% (control). The AD_{CF} values for the PEK and RO diets were comparable to those of the control, whereas those for AO and RD were significantly lower.

Table 8. Apparent digestibility (AD%) of the experimental diets from Paper 1. PEK—PEKILO®, AO—*Aspergillus oryzae*, RD—*R. delemar,* RO—*R. oligosporus,* and Control—control diet.

	Control	PEK	AO	RO	RD	Pooled SEM [1]	*p*-Value [2]
Dry matter	83.3^{a}	76.0^{b}	67.8^{c}	65.2^{c}	65.1^{c}	1.698	<0.0001
Crude protein	91.2^{a}	89.5^{a}	81.3^{b}	79.5^{bc}	76.8^{c}	0.4929	<0.0001
Crude fat	95.3^{a}	94.3^{ab}	93.1^{b}	94.8^{ab}	90.5^{c}	0.7078	<0.0001
Essential amino acids							
Arginine	96.4^{a}	95.6^{a}	91.5^{b}	89.6^{bc}	88.9^{c}	0.6197	<0.0001
Histidine	92.4^{a}	92.3^{a}	84.8^{b}	81.1^{c}	81.0^{c}	0.9101	<0.0001
Isoleucine	94.3^{a}	93.0^{a}	86.3^{b}	81.9^{c}	81.5^{c}	0.9502	<0.0001
Leucine	94.9^{a}	93.7^{a}	87.4^{b}	84.0^{c}	83.4^{c}	0.9121	<0.0001
Lysine	92.5^{a}	93.0^{a}	87.2^{b}	82.9^{c}	82.8^{c}	0.8846	<0.0001
Methionine	93.8^{a}	93.1^{a}	87.0^{b}	84.7^{bc}	83.4^{c}	1.002	<0.0001
Threonine	92.0^{a}	90.8^{a}	82.2^{b}	80.4^{b}	78.7^{b}	1.346	<0.0001
Valine	94.1^{a}	92.7^{a}	86.1^{b}	81.4^{c}	81.2^{c}	1.053	<0.0001
Total amino acids	93.6^{a}	92.6^{a}	86.3^{b}	83.0^{c}	82.9^{c}	0.9763	<0.0001

[1] Pooled standard error of the mean. [2] Significance of one-way ANOVA. Values within the same row that bear different superscript letters are significantly different according to Tukey's multiple comparison test ($p \leq 0.05$).

In paper 2, the AD (%) of the diets is shown in Table 9. No significant differences were detected among the inclusion levels for AD_{DM}. AD_{Ash} differed significantly overall ($p = 0.0291$); however, pairwise comparisons revealed no significant differences between individual treatment groups. Both linear ($p = 0.0013$) and quadratic ($p = 0.0072$) trends were observed for AD_{Ash} across inclusion levels. For AD_{CP}, no significant differences were found among groups, although a significant linear relationship was detected across inclusion levels ($p_{linear} = 0.0254$).

Table 9. Apparent digestibility (AD%) of the different diets in Paper 2. D_0 – control (0% *P. variotii* inclusion), D_5 – 5% *P. variotii* inclusion, D_{10} – 10% *P. variotii* inclusion, D_{20} – 20% *P. variotii* inclusion and D_{30} - 30% *P. variotii* inclusion.

Parameters	D_0	D_5	D_{10}	D_{20}	D_{30}	SEM	p-value	p-linear	p-quadratic	AIC
Dry matter	84.11	84.2	83.04	84.15	82.04	0.63	0.1319	0.0691	0.1457	L
Ash	46.72^a	47.93^a	49.71^a	54.17^a	53.44^a	1.60	0.0291	0.0013	0.0072	L
Crude protein	94.07	94.24	93.83	93.43	93.32	0.32	0.2495	0.0254	0.0813	L

Different superscript letters within a row indicate statistically significant differences between means ($p < 0.05$). *P*-value refers to the ANOVA, whereas p-linear refers to the linear regression model and p-quadratic refers to the quadratic regression model. AIC stands for Akaike information criterion, which is used to decide which model describes the dataset better.

5.4. Growth parameters

In Paper 1, there were no significant differences in weight gain % between the groups fed the various test diets and the control group fed the control diet. However, the corrected FCR was significantly higher in the RO-fed group compared to the control, while no differences were observed among the other treatments. Feed intake values for the different diets are also presented in Table 10, showing no significant variations among the groups.

Table 10. The corrected FCR, weight gain % (WG), SGR (%/day), and feed intake (g/tank) of the fish fed different experimental diets. The novel ingredients in the different diets are PEK—PEKILO®, AO—*Aspergillus oryzae*, RD—*Rhizopus delemar*, RO—*Rhizopus oligosporus*, and Control—control diet.

	Control	PEK	AO	RO	RD	Pooled SEM [1]	p-value [2]
WG (%)	95.5	109.7	101.6	96.9	110.7	9.067	0.3638
Corrected FCR	0.8^b	0.8^{ab}	0.9^{ab}	1.0^a	0.9^{ab}	0.0513	0.0257
SGR (%/day)	1.52	1.68	1.59	1.54	1.69	0.1014	0.3618
Feed intake (g/tank)	948.97	1090.13	1134.80	997.10	1081.67	105.3	0.4387

[1] Pooled standard error of the mean, [2] Significance of one-way ANOVA. Values in the same row with different superscripts indicate significant differences as determined by Tukey's multiple comparison test ($p \leq 0.05$).

The results for growth parameters and body indices from paper 2 are presented in Table 11. There were no significant differences in initial weight among treatments. However, the mean final weight was significantly lower in the D_{30} group compared with all other diets. A significant negative quadratic relationship was found between inclusion level and final weight ($p_{value} = 0.0062$; $p_{linear} = 0.0219$; $p_{quadratic} = 0.0019$). Similarly, weight gain (%) was significantly lower in D30 compared to the control, D10, and D20 groups. A significant negative linear and quadratic relationship was also observed between *P. variotii* inclusion level and weight gain (%) ($p_{value} = 0.0127$; $p_{linear} = 0.0149$; $p_{quadratic} = 0.0037$). The specific growth rate (SGR) followed a similar trend, being significantly lower in D30 than in the other treatments. A significant negative linear relationship was detected between *P. variotii* inclusion level and SGR ($p_{value} = 0.0076$; $p_{linear} = 0.0315$; $p_{quadratic} = 0.0071$). Feed intake was also significantly lower in

D30 compared to all diets except D5, showing a significant quadratic relationship with increasing *P. variotii* levels (p_{value} = 0.0127; p_{linear} = 0.0149; $p_{quadratic}$ = 0.0037). The corrected feed conversion ratio (FCR) did not differ significantly among treatments. However, the Viscerosomatic Index (VSI) increased with inclusion level, showing a significant positive linear correlation (p_{value} = 0.0173; p_{linear} = 0.0065; $p_{quadratic}$ = 0.0283). Although the Hepatosomatic Index (HSI) did not differ significantly between groups, a significant positive linear relationship was observed with inclusion level (p_{value} = 0.2403; p_{linear} = 0.0179; $p_{quadratic}$ = 0.0618).

Table 11: Growth performance of rainbow trout fed different experimental diets in paper 2. Footnote D_0 – control (0% *P. variotii* inclusion), D_5 – 5% *P. variotii* inclusion, D_{10} – 10% *P. variotii* inclusion, D_{20} – 20% *P. variotii* inclusion and D_{30} - 30% *P. variotii* inclusion. The sample size (n) is 15 for all the parameters.

Parameters	D_0	D_5	D_{10}	D_{20}	D_{30}	SEM	p-value	p-linear	p-quadratic	AIC
Initial body weight (g)	42.8	43.1	43.5	43.6	42.5	0.3	0.0579	0.6913	0.0089	Q
Final body weight (g)	233.2^{a}	231^{a}	238.1^{a}	240.6^{a}	190.1^{b}	7.88	0.0062	0.0219	0.0019	Q
Weight gain (%)	445.7^{a}	434.8^{ab}	447^{a}	451.4^{a}	347.1^{b}	18.64	0.0127	0.0149	0.0037	Q
SGR (% day^{-1})	2.2^{a}	2.2a	2.2^{a}	2.2a	1.9b	0.05	0.0076	0.0315	0.1768	L
Feed intake (g tank^{-1})	4115.13^{a}	3957.35^{ab}	4192.2^{a}	4297.71^{a}	3201.02^{b}	183.72	0.0119	0.0944	0.0246	Q
Corrected FCR	0.72	0.71	0.72	0.73	0.73	0.01	0.6436	0.3984	0.5249	L
VSI	10.70^{b}	10.93^{ab}	12.07^{ab}	11.32^{ab}	12.36^{a}	0.32	0.0173	0.0065	0.0283	L
HSI	1.21	1.24	1.33	1.33	1.45	0.07	0.2403	0.0179	0.0618	L

Different superscript letters within a row indicate statistically significant differences between means ($p < 0.05$). *P*-value refers to the ANOVA, whereas p-linear refers to the linear regression model and p-quadratic refers to the quadratic regression model. AIC stands for Akaike information criterion, which is used to decide which model describes the dataset better.

5.5. Nutrient retention

The results of nutrient retention from Paper 2 are presented in Table 12. Nitrogen retention was significantly lower in the D_{30} group compared with all other treatments. A significant negative quadratic correlation was observed between inclusion level and nitrogen retention (p_{value} = 0.0076; p_{linear} = 0.0397; $p_{quadratic}$ = 0.0111). Similarly, energy retention was significantly reduced in D_{30}, with a significant negative quadratic relationship associated with increasing inclusion levels. Crude fat retention was significantly lower in D_{30} compared with D_{20}, though not significantly different from the other diets. A significant negative quadratic correlation was also detected (p_{value} = 0.0324; p_{linear} = 0.152; $p_{quadratic}$ = 0.0132). Phosphorus retention was significantly lower in D_{30} compared with all diets except D_5, and a significant negative quadratic relationship was observed with inclusion level (p_{value} = 0.0173; p_{linear} = 0.0182; $p_{quadratic}$ = 0.0164). Magnesium and potassium retention levels were also significantly reduced in D_{30} compared with the other treatments. Both minerals showed significant negative quadratic correlations with increasing inclusion levels (Mg: p_{value} = 0.0014; p_{linear} = 0.0262; $p_{quadratic}$ = 0.0064; K: p_{value} = 0.0003; p_{linear} = 0.0201; $p_{quadratic}$ = 0.0037).

Table 12: Nutrient retention (in (g/fish) unless specified otherwise) of fish fed the different experimental diets with increasing levels of *P. variotii.* D_0 – control (0% *P. variotii* inclusion), D_5 – 5% *P. variotii* inclusion, D_{10} – 10% *P. variotii* inclusion, D_{20} – 20% *P. variotii* inclusion and D_{30} - 30% *P. variotii* inclusion.

Nutrient	D_0	D_5	D_{10}	D_{20}	D_{30}	p-value	p-linear	p-quadratic	AIC
Nitrogen	86.88^a	83.89^a	86.87^a	88.40^a	66.50^b	0.0076	0.0397	0.0111	Q
Energy (kJ $fish^{-1}$)	49.11^a	49.27^a	50.59^a	52.21^a	38.29^b	0.0073	0.0867	0.0095	Q
Crude fat	77.23^a	80.66^a	82.27^a	83.62^a	62.03^b	0.0324	0.152	0.0132	Q
Phosphorous	23.33^a	21.87^{ab}	22.60^{ab}	22.66^{ab}	17.77^b	0.0173	0.0182	0.0164	Q
Magnesium	1.6^a	1.52^a	1.59^a	1.61^a	1.23^b	0.0014	0.0262	0.0064	Q
Potassium	19.26^a	18.28^a	19.17^a	19.41^a	14.61^b	0.0003	0.0201	0.0037	Q
Calcium	22.14	21.06	20.39	20.57	17.32	0.3142	0.0426	0.1139	L
Sodium	4.53^a	4.50^a	4.67^a	4.57^a	3.55^b	0.0023	0.0251	0.0014	Q
Sulphur	10.15^a	9.76^a	10.27^a	10.31^a	7.70^b	0.0011	0.0295	0.004	Q

Different superscript letters within a row indicate statistically significant differences between means ($p < 0.05$). *P*-value refers to the ANOVA, whereas p-linear refers to the linear regression model and p-quadratic refers to the quadratic regression model. AIC stands for Akaike information criterion which is used to decide which model describes the dataset better.

5.6. Histology

The intestinal villi length and goblet cell area in samples from the proximal intestine in paper 2, along with the results from the Ussing chamber experiment, are presented in Table 13. Villi length was significantly shorter in the D_5 group compared to D_{30}. A significant positive linear relationship was also observed between *P.variotii* inclusion level and villi length ($p_{value} = 0.03024$; $p_{linear} = 0.0044$; $p_{quadratic} = 0.0196$). The goblet cell area did not differ significantly among the dietary treatments.

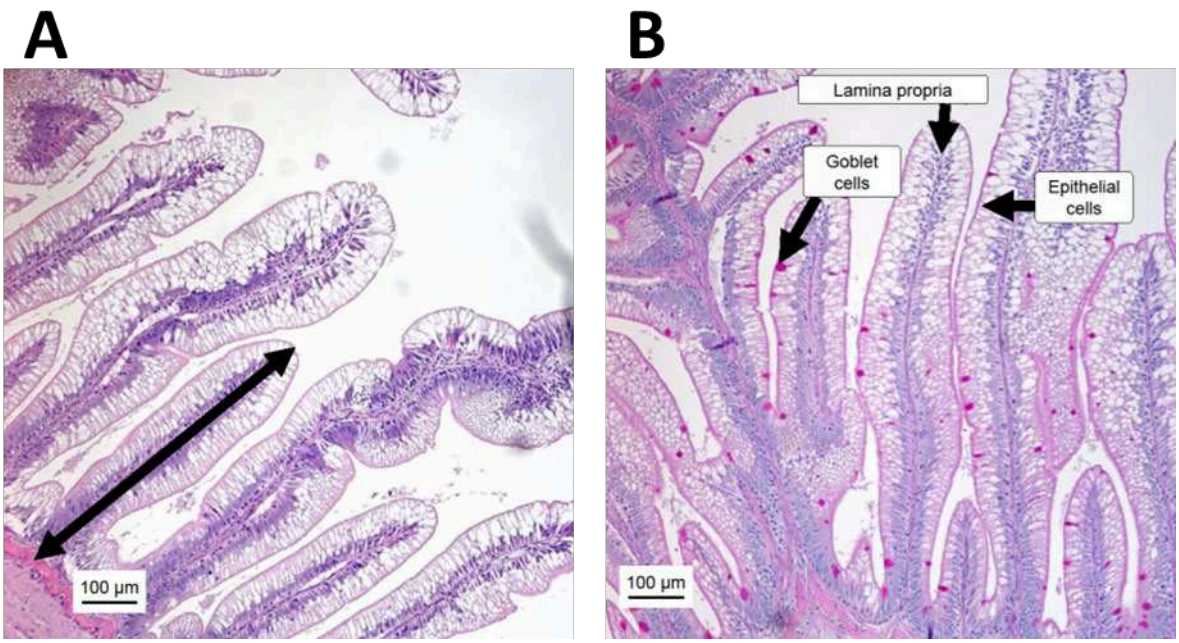

Figure 6. Representative histological measurements of A) Villi length (indicated by black, double-headed arrow) and B) goblet cells and epithelial cells of villi (indicated by black arrows).

Table 13. Gut health parameters of fish fed the different experimental diets with increasing levels of *P. variotii.* D_0 – control (0% *P. variotii* inclusion), D_5 – 5% *P. variotii* inclusion, D_{10} – 10% *P. variotii* inclusion, D_{20} – 20% *P. variotii* inclusion and D_{30} - 30% *P. variotii* inclusion.

	D_0	D_5	D_{10}	D_{20}	D_{30}	SEM	p-value
Villi length (µm)	857.9^{ab}	825.7^{b}	904^{ab}	934.9^{ab}	939.8^{a}	23.57	0.0302
Goblet cell area (%)	3.3	3.5	3.6	3.3	3.6	0.34	0.9104

Different superscript letters within a row indicate statistically significant differences between means ($p < 0.05$). *P*-value refers to the ANOVA, whereas p-linear refers to the linear regression model and p-quadratic refers to the quadratic regression model. AIC stands for Akaike information criterion which is used to decide which model describes the dataset better.

5.7. Intestinal physiology

The short-circuit current (SCC) of the proximal intestine did not differ significantly among the dietary groups; however, a significant positive linear correlation was observed with increasing *P. variotii* inclusion levels ($p_{value} = 0.0587$; $p_{linear} = 0.0111$; $p_{quadratic} = 0.0275$). The SCC of the distal intestine showed no significant variation between the different experimental diets. The transepithelial potential (TEP) of the proximal intestine was significantly lower in D_{30} compared with the control group. A significant negative linear relationship was also observed with increasing inclusion levels ($p_{value} = 0.0024$; $p_{linear} \leq 0.0001$; $p_{quadratic} = 0.0004$). In contrast, TEP in the distal intestine did not differ significantly among treatments. The transepithelial resistance (TER) of the proximal intestine showed no significant differences between dietary groups, and no clear relationship was observed with inclusion level. However, in the distal intestine, TER was significantly lower in the control group compared with D_{20}, with a significant positive quadratic relationship identified between increasing inclusion levels.

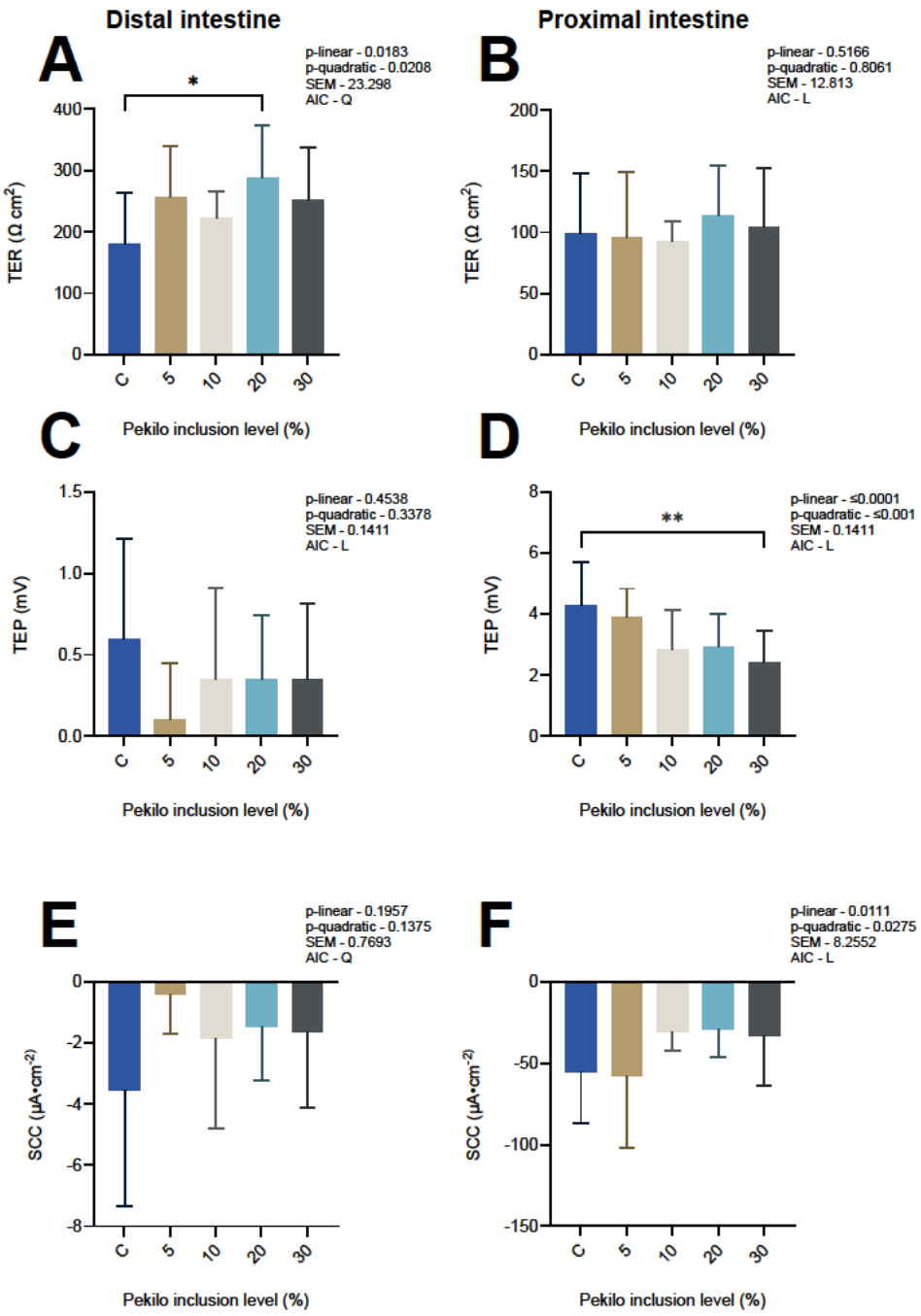

Figure 7. Ussing chamber results for transepithelial resistance (TER) of A. Distal and B. Proximal; transepithelial potential (TEP) of C Distal and D. Proximal and Short circuit current (SCC) of E. Distal and F. Proximal intestine. The groups that were tested significantly different from each other using ANOVA post-hoc were denoted by * for $p \leq 0.05$ and by ** for $p \leq 0.005$. p-linear and p-quadratic were used to denote the significance of the regression models. SEM denotes the standard error over mean for the values, and AIC – L or Q to determine the model that best describes the distribution based on the AIC value.

6. Discussion

Aquaculture is the fastest-growing food sector in the world [67]. Over the past few years, the sector has faced significant challenges in acquiring sustainable protein sources to feed farmed fish, including rainbow trout. To address this, there is a growing interest in microbial ingredients that are produced by valorising waste streams. In this thesis, several microbial ingredients such as *A. oryzae, R. oligosporus, R. delemar* and *P. variotii* were evaluated for their potentials as alternative protein sources for rainbow trout feeds. In the first part of the thesis (Paper 1), the digestibility of the test microbial ingredients was studied. The second (Paper 2) part of the thesis focused on evaluating the dietary effect of the test ingredients on growth performance and health effects of rainbow trout.

6.1. Chemical composition of the ingredients (Paper 1)

To formulate the diets for the digestibility and growth performance experiments, the proximate composition of the test ingredients were analyzed. The crude protein (CP) content of the ingredients tested were quite high. The CP content of the ingredients ranged from 41% to 63%. Specifically, RO had a CP value of 48.7%, which was slightly higher than 47.9% as observed by Langeland M. et al. [31]. The CP content of *P. variotii* in the present study was slightly higher (66.7%) than that reported by Hooft et al. [47] (62.5%). Conversely, the CP values of AO (44.1%) and RD (49.3%) were lower than the ranges reported by Karimi et al. [68], which were 48.6–53.7% for AO and 48.6–53.2% for RD. The observed variation in CP content may result from differences in substrates, extraction methods, fungal strains, or cultivation conditions [69-71]. Nevertheless, *P. variotii* has the highest protein content and the content is comparable to that of the commonly used feed ingredients, such as fish meal and soy protein concentrate that have a protein content ranging from 62% to 70% [72].

The fat content of the ingredients is not a major factor, as the required lipid levels are typically adjusted later through the addition of oils. Nevertheless, fat content can influence the extrusion process and hence a lower fat content is preferred. Because lipids act as natural lubricants, excessively high fat levels can lead to instability during extrusion [73, 74], emphasizing the need to maintain their concentrations at minimal levels. Among the tested ingredients, only AO exhibited a relatively high fat content (12.5%), while the other ingredients contained considerably lower levels. This is consistent with the physical pellet quality analysis, where AO showed a markedly lower expansion rate compared with the control and *P. variotii* diet pellets.

The amino acid composition of the different ingredients was compared using chemical score and essential amino acid index (EAAI) as shown in Figures 4 and 5, respectively. The EAAI value of soybean meal in the present study was 93% when fish meal was used as the reference protein, which closely aligns with the findings of Agboola et al. [15]. In contrast, the EAAI values of the tested ingredients reported in that study ranged from 67 to 79, which are higher than the lower range of 30 to 63 observed here. This suggests that, compared with multicellular fungi, yeasts may possess an amino acid composition more compatible with the nutritional requirements of salmonids. Nevertheless, filamentous fungi remain advantageous over single-celled fungi due to their relatively high crude protein content and the practicality of large-scale biomass recovery from the culture medium.

The major limiting amino acids for salmonid feed are methionine and lysine [75]. The levels of individual amino acids can be compared with fishmeal using the chemical score. The chemical score was lower for methionine in all the tested ingredients, indicating that methionine levels could potentially be a drawback as a protein source to replace fish meal and soy at higher inclusion levels for rainbow trout feed. Karimi et al. [76] reported that the methionine content of multicellular fungi

derived from pure cultures was lower than that of fish meal, although neither the chemical score nor the EAAI was calculated. In the present study*, P. variotii* exhibited a lysine chemical above 100, indicating a higher lysine concentration in its crude protein compared with fish meal. In contrast, the lysine chemical for RO was below 50%, suggesting that lysine may be a limiting amino acid in this ingredient. Karimi et al. [76] also reported that the lysine content of multicellular fungi cultivated on pure substrates was comparable to that of fish meal.

6.2. Physical pellet quality (Paper 1 and Paper 2)

Physical pellet quality is crucial for rainbow trout feed, as it influences feed intake, water stability, and nutrient retention. Durable, well-formed pellets minimize feed wastage and nutrient leaching, ensuring consistent nutrient delivery and efficient growth performance [50].

In the first experiment (Paper 1), AO feed has a significantly lower pellet width and consequently expansion, compared with control and *P. variotii,* which could be attributed to the higher fat levels (12.5%) in the AO ingredient. Hansen et al. [77] observed that expansion after extrusion decreased with an increase in non-soluble polysaccharide (NSP) fractions. This could also support the reason behind significantly lower expansion in RO, RD and AO compared with *P. variotii* and control. This hypothesis is further supported by the sum of analysed nutrients: CP, CF and ash content, which are 642, 537, 638 and 802 g/kg DM for AO, RO, RD and *P. variotii*, respectively. This suggests that the carbohydrate fractions, and possibly the fiber (NSP) fractions are much higher in AO, RO and RD.

In the second experiment (Paper 2), the experimental diets, prepared using varying levels of *P. variotii,* were also examined for pellet width and expansion. Results showed that neither of the tested variables were affected by increasing inclusion levels of the microbial ingredient *P. variotii.* This is in contrast with Hooft et al. [47] where addition of *P. variotii* at different inclusion levels affected the various pellet quality parameters such as pellet expansion, sinking velocity etc.

High water stability is desirable in rainbow trout feeds, as poor stability, especially under certain environmental conditions, can lead to digestive issues, such as oil belching and abdominal distention syndrome [50, 57, 78]. The water stability of a diet is largely influenced by the composition and properties of its ingredients [50, 79]. Among the tested diets in Paper 1, the *P. variotii* -containing diet exhibited the highest water stability and the highest apparent digestibility coefficients of protein (AD_{CP}). This observation agrees with earlier studies reporting a positive relationship between feed stability and digestibility parameters such as AD_{CP} and AD_{CF} [57]. The water stability index of the *P. variotii* diet did not differ significantly from that of the control, even after 60 minutes of immersion, which can be considered a favorable outcome. This suggests that the feed is likely to remain intact during consumption, thereby minimizing the risk of digestibility-related issues. In contrast with our study, Hooft et al. [47] also reported improved water stability following the inclusion of the microbial ingredient *P. variotii* whereas the microbial inclusions in the present study resulted in a reduction in feed stability. This discrepancy may stem from differences in inclusion levels and ingredient composition. Because the diets in the present experiment were formulated based on a 70:30 ratio, the starch content, which affects binding, may have varied, thereby influencing the overall structural integrity and stability of the pellets. In the second experiment (Paper 2), increasing the inclusion levels of *P. variotii* improved the water stability index of the diets at different timepoints. This is in agreement with Hooft et al. [47], who also observed increasing inclusion levels to improve the water stability index. In the second experiment (Paper 2), the reduced pellet durability at 30% inclusion suggests that the transportation losses could be high in the diet [80]. The lower sinking velocity observed in the 5%

P. variotii inclusion level diet may warrant further investigation but this did not affect the overall pellets sinking rate.

6.3. Apparent digestibility (Paper 1 and 2)

The apparent digestibility of an ingredient can be affected by different factors, such as proximate composition, cell wall fraction, water temperature, sampling method. [40, 77]

In the first experiment (Paper 1), the dietary AD_{DM} as reported in Table 8 was significantly higher in the control diet compared with the other treatments. Among the diets containing microbial ingredients, *P. variotii* showed the second-highest AD_{DM}, while AO, RD, and RO exhibited statistically similar values. The ingredient AD_{DM} (Table 7) for RD and RO was 23.6% and 24.1%, respectively, suggesting limited digestibility. This reduced digestibility may be associated with the presence of indigestible cell wall materials in the microbial biomass or residual fermentation substrates [81, 82]. However, it is also possible that the stripping method to collect feces could have led to an underestimation of digestibility [83, 84]. Moreover, repeated handling during stripping has been reported to reduce feed intake and growth in rainbow trout [85], potentially influencing digestibility measurements in this study.

In the second experiment (Paper 2), the AD_{DM} for the different diets is represented in Table 9. There were no significant differences, indicating that the overall digestion and absorption of feed components were similar across treatments. This suggests that the inclusion of different ingredients did not markedly affect the ability of the fish to utilize the total dry matter in the diets. The AD_{DM} observed in Hooft et al. [47] was lower than the ones observed in this study, whereas the AD_{DM} observed in Dahlberg [44] was higher than what was found in this study.

In Paper 1, despite the potential sources of digestibility variation mentioned above, the AD_{CP} in the control diet was 91.2%, which is similar to values reported for fish-meal-based diets in salmonids, typically ranging from 82.7% to 92.1% [31, 48, 82]. As mentioned earlier, AO, RO and RD potentially have higher carbohydrate and fiber fractions which rainbow trout are unable to utilize. This could be one of the reasons for the lower dietary and ingredient AD_{CP} compared with control and *P. variotii*, respectively. The lower protein digestibility observed in diets containing fungal ingredients may also be related to the structural complexity of the fungal cell wall [15, 86, 87].

In Paper 2, dietary AD_{CP} did not seem to differ between the different diets. Compared with the results from Paper 1, where the AD_{CP} was 89.5%, which was slightly lower than what was observed in this study. This could have been due to differences in feces sampling method and/or the composition of the diets. Hooft et al. [47] noted that dietary AD_{CP} ranged from 86.8% to 89.8% which are lower than the values observed in Paper 2. On the other hand Dahlberg [44] observed that the AD_{CP} ranged from 91.2% to 92.8% in their study which was similar to the AD_{CP} observed in Paper 2. These differences could be due to the nature of the substrate that was used to grow the microbial ingredient.

Regarding the apparent digestibility of ash and amino acids, in Paper 1, the AD_{AA} of methionine and lysine were much higher in *P. variotii* compared with the other ingredients. This indicates higher amino acid absorption and subsequently lesser nutrients emitted into the surroundings. On the other, hand, in Paper 2, there is a linear increase in AD_{Ash} which could indicate an increased mineral availability in the test ingredients.

6.4. Growth performance (Paper 1 and 2)

In paper 1, no significant differences in weight gain were observed among the dietary treatments, indicating that the inclusion of fungal biomass did not affect growth performance. This observation aligns with the findings of Vidakovic et al. [48], who also reported no variation in growth at high inclusion levels of fungal ingredients. In contrast, Dahlberg [44] observed differences in weight gain after only 4–5 weeks of feeding, suggesting that the duration of the present trial may have been too short to reveal potential growth effects. Nonetheless, the absence of significant differences in growth can be regarded as a positive result, as it demonstrates that replacing conventional ingredients with microbial biomass did not impair performance. Long-term studies using nutritionally balanced diets are needed to better assess growth responses to these ingredients.

All FCR values were below 1, indicating efficient feed utilization. However, RO had a significantly higher FCR than the other diets, suggesting that nutrients in this treatment were used less efficiently. Total feed intake did not differ significantly among treatments, implying that even at 30% inclusion levels, the test ingredients did not negatively influence feed palatability.

Paper 2 demonstrated that *P. variotii* protein can effectively replace fish meal and soy protein concentrate as a dietary protein source in rainbow trout diets up to an inclusion level of 20%. Beyond this threshold, however, growth performance declined significantly, as evidenced by the lower mean final body weight and percentage weight gain at 30% inclusion after the nine-week feeding period. These findings are consistent with those of Hooft et al. [47], who reported no adverse effects on growth in Atlantic salmon when up to 20% of the dietary protein was replaced with *P. variotii* over a similar experimental duration. In contrast, Dahlberg [44] observed reduced growth in rainbow trout at inclusion levels of 20% and 30% after 44 days, suggesting that the tolerance threshold may vary with diet composition, species, or experimental conditions.

A quadratic response was observed for weight gain, showing a numerical increase up to 20% inclusion followed by a decline at 30%, indicating a potential upper limit for *P. variotii* inclusion. Further studies with intermediate inclusion levels between 20% and 30% would be valuable for pinpointing the optimal range for growth performance in rainbow trout. The diets containing 30% *P. variotii* also had lower crude fat and gross energy contents than the other treatments. Given that dietary lipids can exert protein-sparing effects in rainbow trout [88] the reduced nitrogen retention observed at this inclusion level may partly reflect the lower lipid content. At the same time, the decreased fat level increases the dietary protein-to-energy (DP/DE) ratio, which is beneficial for optimizing growth and feed efficiency while minimizing nutrient losses, including phosphorus emissions [89].

Research on *P. variotii* in terrestrial animals supports its potential as a protein source. Näsi [90] reported that substituting soybean meal with *P. variotii* did not affect the digestibility of dry matter, crude protein, or crude fat in pigs. Similarly, in another study, Näsi [91] found no significant changes in egg production or feed conversion ratio in poultry when soybean and fish meal were replaced with *P. variotii*, although feed intake increased at higher inclusion levels. In pigs, Järvinen et al. (1980) observed that dietary inclusion of up to 15% *P. variotii* did not negatively influence growth performance compared to the control diet.

The HSI and VSI values observed in this study are consistent with those reported by Hossain et al. [92], where HSI ranged from 1.13 to 1.24 and VSI from 10.34 to 10.94. A clear linear increase in VSI was

detected with rising inclusion levels of *P. variotii*. This increase may be linked to greater fat accumulation [93, 94] and/or enlargement of visceral organs. The concurrent linear rise in HSI with increasing *P. variotii* inclusion supports the likelihood that higher visceral fat deposition contributed to the elevated VSI values. Generally, a lower VSI is preferable, as it reflects a higher proportion of edible flesh relative to viscera.

6.5. Nutrient retention (Paper 2)

The retention data from Paper 2 revealed patterns similar to those observed for growth responses, with nitrogen and energy retention both showing a quadratic trend in relation to *P. variotii* inclusion level. Retention was highest at moderate inclusion levels (around 20%) and declined at 30%. Lower nitrogen retention at the highest inclusion level may reflect reduced nutrient digestibility, suboptimal amino acid balance, or possible effects on gut function [95]. The enhanced nitrogen retention at lower inclusion levels may be linked to the high nucleic acid content of microbial ingredients, which can reduce the need for de novo nucleotide synthesis and thereby spare amino acids for growth, as suggested by Hooft et al. [47]. Similar findings have been reported in other studies using microbial protein sources [24, 96, 97].

Crude fat retention also followed a quadratic trend, peaking at 20% inclusion and declining at 30%. These variations could be related to differences in dietary lipid composition, as rainbow trout more efficiently utilize polyunsaturated than saturated fatty acids [98]. Phosphorus retention was highest in the control diet and decreased at 30% inclusion, again displaying a significant quadratic relationship. Since microbial ingredients lack phytate-bound phosphorus, unlike plant proteins, the reduced phosphorus retention at higher inclusion levels likely reflects a general decline in digestibility. Nevertheless, the potential for certain fungal strains to produce phytase [99] or the inclusion of acidifiers such as formic acid [100] may help improve phosphorus bioavailability in future formulations.

6.6. Intestinal health (Paper 2)

An increase in villus length was observed in fish fed the 30% inclusion diet, which may suggest enhanced digestive capasity and intestinal health. Comparable results have been reported by Hooft et al. [47] and Mensah et al. [101]. The higher nucleotide content of microbial ingredients could contribute to this effect, as nucleotides are known to stimulate intestinal cell proliferation [102, 103]. Other nutrients, such as sodium butyrate and phenylalanine, have also been associated with villus elongation by promoting epithelial growth or triggering growth factor release [51, 104]. Although these mechanisms were not examined in the present study, they warrant further investigation to clarify the underlying cause of villus development. The proportion of goblet cell area did not differ among dietary treatments, and villus morphology remained intact, suggesting that none of the diets induced intestinal inflammation.

Related to gut barrier function, the absence of significant differences in transepithelial resistance (TER) and short-circuit current (SCC) in the proximal intestine indicate stable epithelial integrity and ion transport across diets. However, the lower transepithelial potential (TEP) observed at 30% inclusion, along with its negative correlation with *P. variotii* level, may imply some influence of the ingredient or its components on the intestinal epithelium's electrical properties. In the distal intestine, fish fed the D_{20} diet exhibited higher TER than the control group, which could reflect improved barrier function through reduced paracellular permeability. Since the proximal intestine plays a key role in

digestion and nutrient absorption, any alteration in its function could have long-term implications for feed utilization and growth, although no clear adverse effects were detected within the experimental period.

7. Concluding remarks

Among the microbial ingredients tested in this thesis, *P. variotii* showed the highest digestibility, followed by AO and RO, while RD had the lowest. Its chemical composition revealed the highest crude protein content and the most suitable amino acid profile, supporting its strong nutritional value. Overall, *P. variotii* appears to be a promising alternative protein source for rainbow trout feeds at inclusion levels up to 20%, with minimal to no negative effects on gut health. Although reduced growth and nutrient retention were observed at 30% inclusion—indicating a potential upper threshold—indicators of gut integrity such as TER, SCC, and TEP remained generally stable across diets, suggesting that epithelial function was not compromised and that the higher TER in the distal intestine at 20% inclusion may even reflect improved barrier properties. Taken together, the results indicate that a 20% inclusion level of *P. variotii* offers the best balance among pellet quality, growth performance, and gut health.

8. Future perspectives

Microbial ingredients represent promising alternative protein sources for aquafeeds. They can be produced using a wide range of substrates, grown year-round under controlled conditions, and cultivated without competing for arable land. The microbial ingredients evaluated in this thesis were all derived from side-stream substrates, potentially enhancing their sustainability.

Although the digestibility of some ingredients tested in Paper 1 was lower than others, there remains potential for improvement through advances in processing and refining technologies. A noteworthy observation from the study was that feed intake for all microbial ingredients at a 70:30 replacement ratio exceeded that of the control diet, suggesting a possible enhancement of feed palatability. Further investigation is warranted to confirm this effect and to explore the potential use of these ingredients as palatability enhancers. The essential amino acid index of all tested ingredients was considerably lower than that of fish meal and soy protein concentrate, indicating the need to optimize the amino acid composition of microbial biomass, possibly through modification of growth substrates. This can be adjusted during feed formulation, either by using complementary protein sources or by use of crystalline amino acids such as methionine to end up with the optimal amino acid profile.

In Paper 2, up to 30% *P. variotii* did not negatively impact the growth performance or gut health of rainbow trout. Given its established potential for large-scale production, *P. variotii* appears suitable as an alternative protein source. An interesting finding was the increase in villus length at the 30% inclusion level. Since greater villus length enhances the absorptive surface area, identifying and isolating the compound(s) responsible for this effect could enable their use as functional feed additives. Furthermore, elucidating the underlying molecular mechanisms, particularly those related to immune responses, would provide valuable insights into the biological effects of microbial ingredients in aquafeeds.

To move forward, it is essential to explore the limits of what is possible and to explore the limits of what is possible and determine the point at which practical application becomes feasible. Evaluating combinations of alternate protein ingredients at varying inclusion levels could, therefore, be a natural next step moving forward to identify an optimal feed formulation for rainbow trout.

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