



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

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

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

## INTRODUCTION

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

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

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

## MATERIALS AND METHODS

### Fish Husbandry

Juvenile rainbow trout were purchased from Vilstena Fiskodling AB, Fjärdhundra, Sweden, and the experiment was carried out in the Aquatic Facility, Center of Veterinary Medicine and Animal Science, Swedish University of Agricultural Sciences, Uppsala, Sweden. A total of 300 fish (average weight  $127.8 \pm 19.8$  g) were randomly and evenly distributed between 15 oval experimental tanks (200 L) and reared in a 12-h light cycle (08.00–20.00 h). The experimental tanks were equipped with a partial recirculation system and supplied with fresh tap water at  $3 \text{ L min}^{-1}$ .

All fish were judged to be healthy, with no visible signs of injuries detected on skin, gills, or fins. Each experimental tank was connected to a waste feed and feces collection system. Temperature during the whole experiment was  $11 \pm 1^\circ\text{C}$  and oxygen level was  $8 \pm 2 \text{ mg/L}$  (HQ40D Portable Multi Meter, Hach, Loveland, CO, United States). The fish were acclimatized for 10 days on a commercial diet (Biomar EFICO ENVIRO 920 ADVANCE, 2% of body weight once a day prior to the experiment. The experiment was performed in compliance with laws and regulations on procedures and experiments on live animals in Sweden, which are overseen by the Swedish Board of Agriculture (diary number: 5.8.18-16347/2017).

### Production of *Neurospora intermedia* Biomass

Fungal biomass of *N. intermedia* CBS 131.92 (Centraalbureau voor Schimmelcultures, Netherlands) was produced under semi-continuous cultivation condition at the Swedish Center for Resource Recovery, University of Borås. The fungus was cultivated on complex medium containing 30 g/L glucose and 5 g/L yeast extract as the major carbon and nitrogen source, respectively. Trace elements in the form of  $(\text{NH}_4)_2\text{SO}_4$ ,  $\text{KH}_2\text{PO}_4$ ,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , and  $\text{MgSO}_4 \cdot x\text{H}_2\text{O}$  in concentrations of 7.5, 3.5, 1.0, and 0.75 g/L were added to the cultivation medium to support filamentous fungi growth, using a 26 L capacity bubble column bioreactor (airlift bioreactor converted to bubble column bioreactor by removing the internal loop tube) (Bioengineering, Switzerland). Cultivation was carried out at  $35^\circ\text{C}$  and 1 vvm (volume of air per volume of medium per minute). Cultivation condition parameters and sterilization method were according to Ferreira et al. (2015). To harvest biomass, 75% of the working volume of the reactor (15 L) was harvested twice per day, at 11.00 and 23.00 h. Fresh sterilized cultivation media was added to top up the cultivation broth after harvesting. Harvested broth, containing post-cultivation medium and biomass, was transferred to a cold room and stored at  $4^\circ\text{C}$ . After termination of cultivation, biomass was quickly separated from the culture media using a sieve, washed with distilled water, and dried in an air oven at  $70^\circ\text{C}$ .

### Diets and Feeding

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

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

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

## Sample Collection

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

**TABLE 1** | Dietary composition (g kg<sup>-1</sup> on dry matter basis) of the reference diet (RD), non-preconditioned diet (NPD), and preconditioned (heat-processed) diet (PD).

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

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

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

## Chemical Analysis

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

## Extraction of DNA

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

## Library Preparation and Sequencing

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

## Bioinformatics Analysis

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

<sup>1</sup><http://ccb.jhu.edu/software/FLASH/>

<sup>2</sup>[http://qiime.org/scripts/split\\_libraries\\_fastq.html](http://qiime.org/scripts/split_libraries_fastq.html)

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

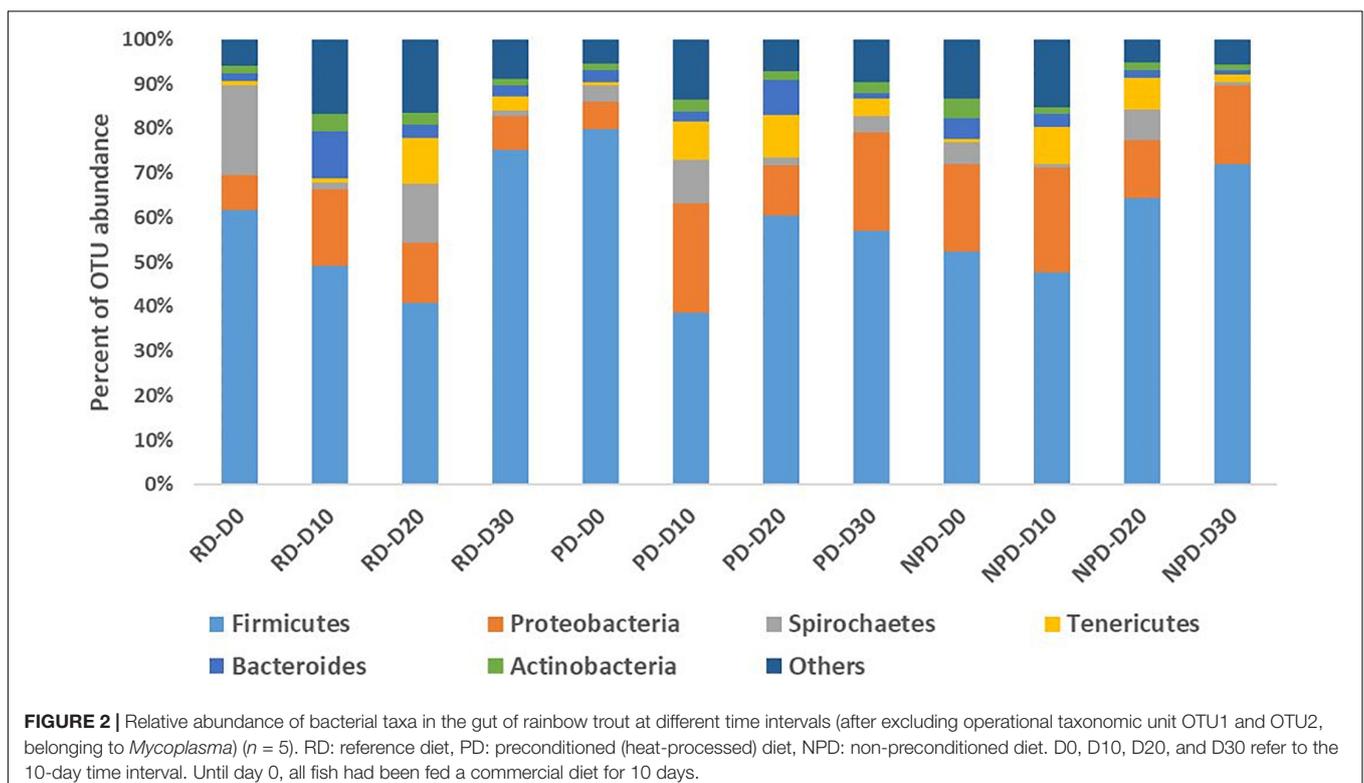
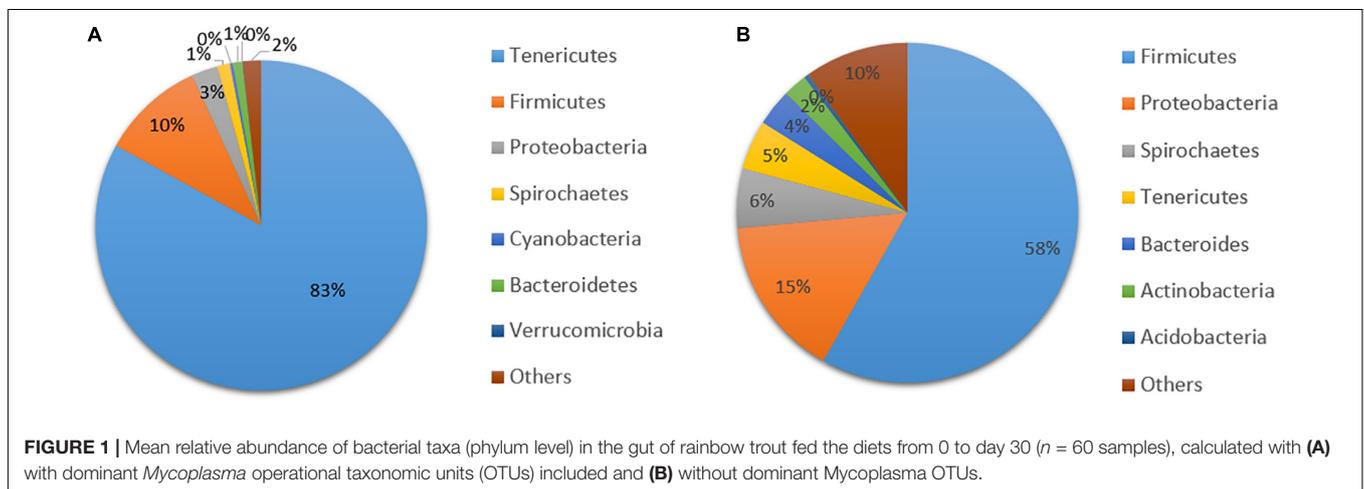
## Statistical Analysis

A linear mixed effect (LME) model (“nlme” package) was used to test for statistically significant differences between

<sup>3</sup><http://drive5.com/uparse/>

<sup>4</sup><http://www.arb-silva.de/>

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



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

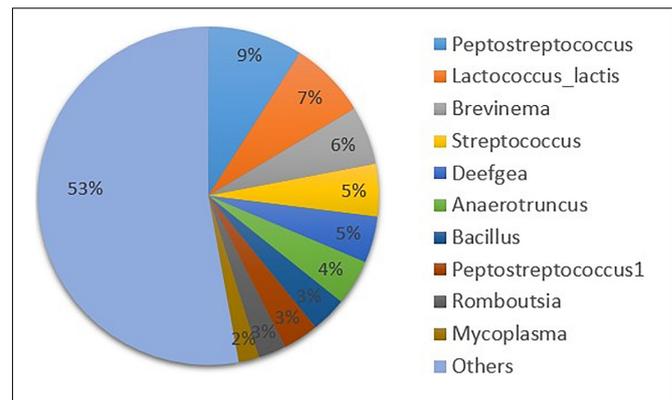
## RESULTS

### Gut Microbial Composition of Rainbow Trout

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

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

Principal coordinate analysis was performed to graphically explore the shift in community structure for different diets after different time intervals (Figures 4A,B). The percentage variation (PoV) explained for axis 1 and 2 when using Bray Curtis index was 25.5 and 10.3%, respectively. For the analysis based on Jaccard's dissimilarity, PoV explained by axis 1 was 13% and by axis 2 was 6.68%. Differences in gut microbial composition at each day (within interval) were analyzed with one-way ANOSIM. The results confirmed that gut microbial composition for the different diets was similar at day 0 and day 30, but dissimilar at day 10 and day 20 (Supplementary Table 1).

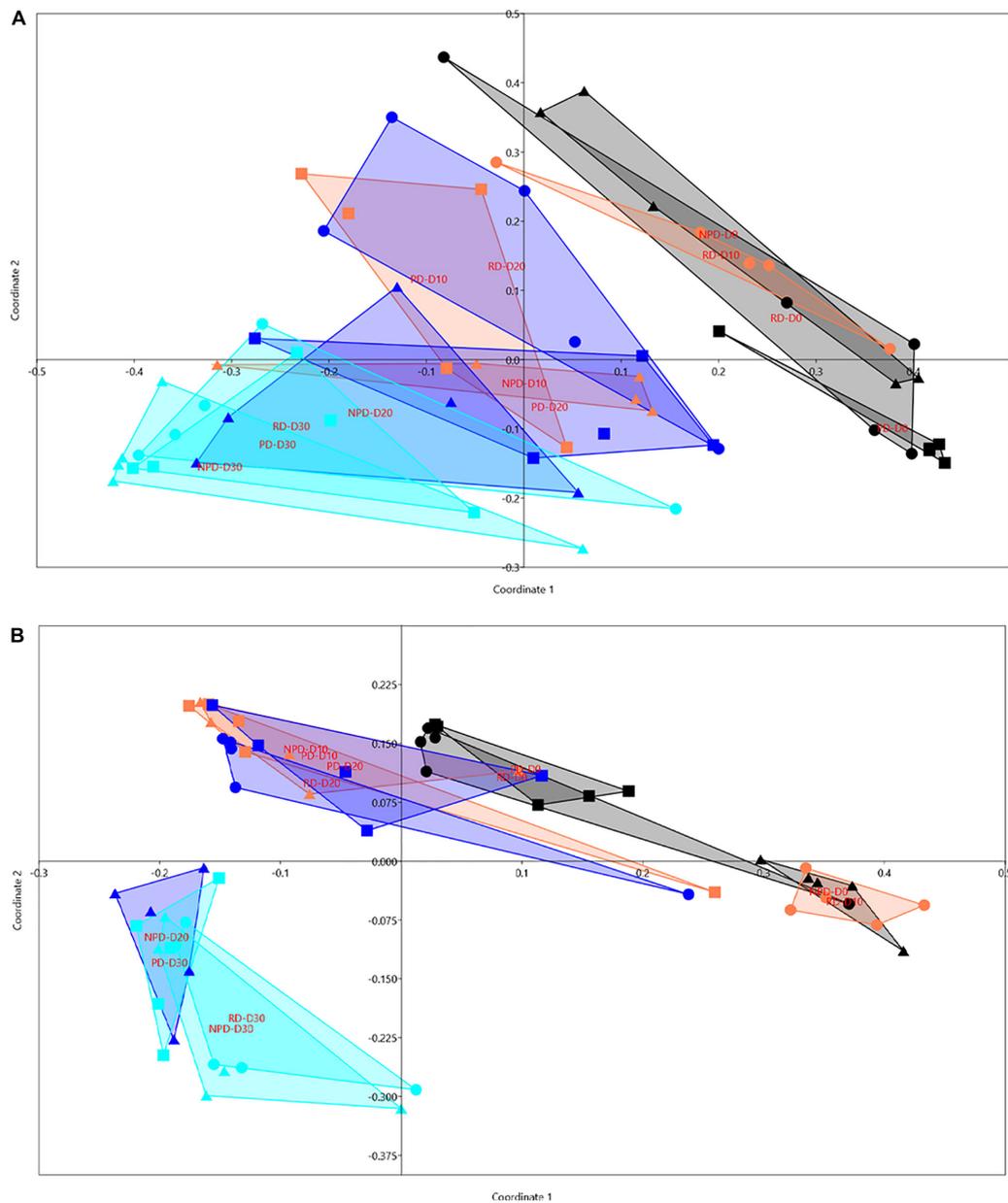


**FIGURE 3** | Mean relative abundance of the top 10 bacterial operational taxonomic units (OTUs) in the gut microbiota of rainbow trout from 0 to 30 days on the commercial and experimental diets ( $n = 60$ ).

Pairwise comparison of the treatment groups showed that they were significantly dissimilar within and between 10-day intervals (Supplementary Table 2). At day 10, the overall microbial composition of the fish gut with diet PD and NPD was different from that with RD. At day 20 there was a difference in microbial composition between NPD and PD, but they did not differ from RD. Over 10-day intervals, there was a temporal change in gut microbial composition with all diets from day 0 to day 10, from day 10 to day 20, from day 20 to day 30, and from day 10 to day 30 (Supplementary Table 2). According to SIMPER analysis, the percentage dissimilarity for the pairwise-compared treatment groups ranged from 64.73 to 82.58%.

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

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



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

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

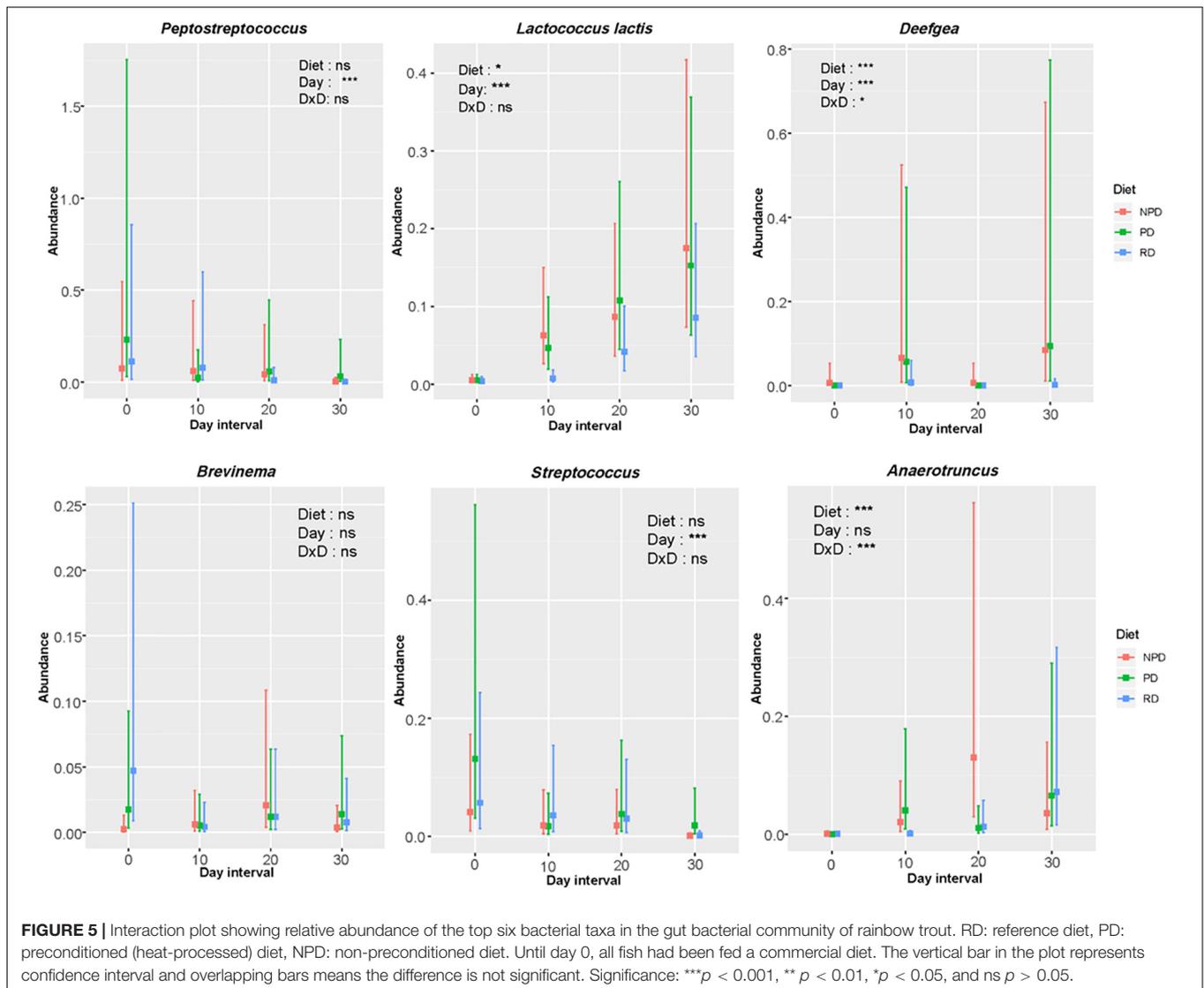
### Growth Performance

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

## DISCUSSION

### Shift in Overall Gut Microbial Composition With Diet and Time

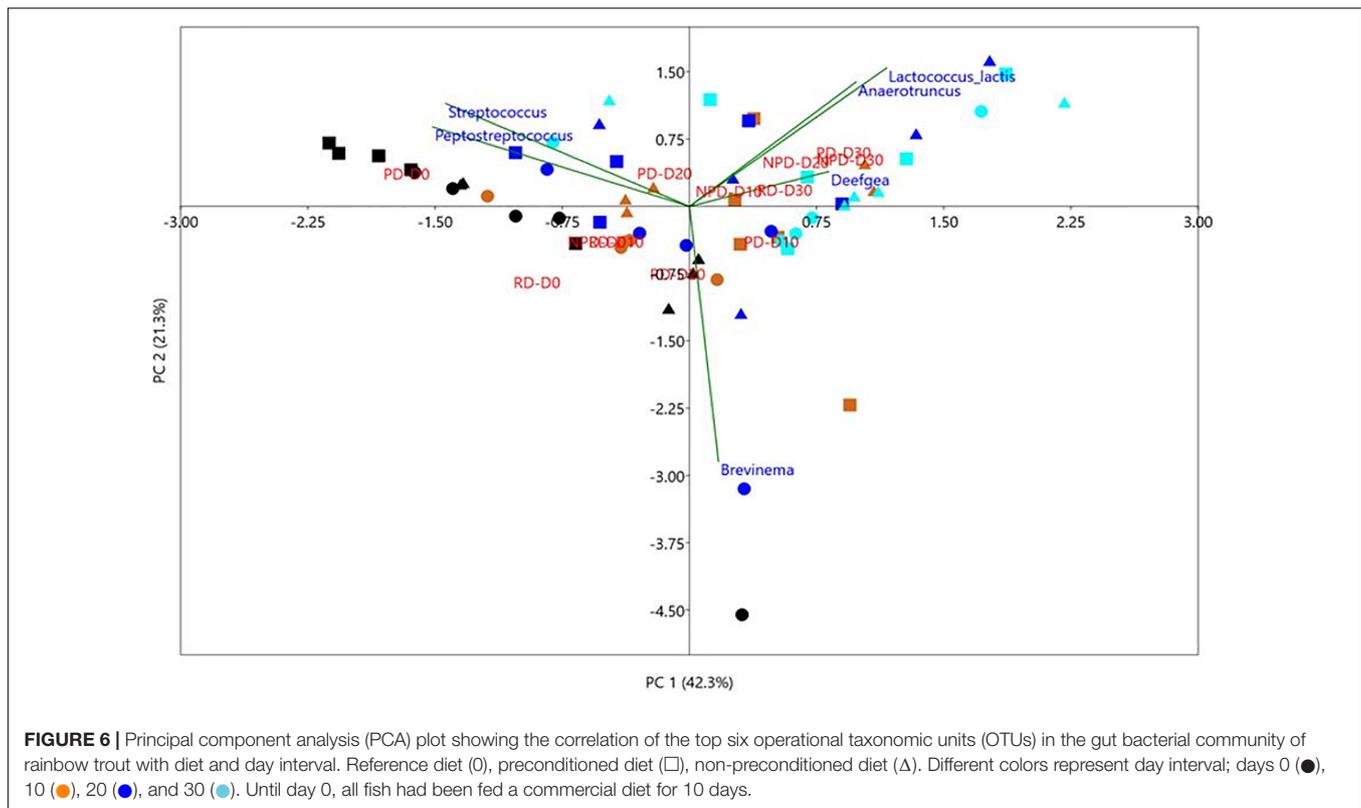
The PCoA, ANOSIM, and SIMPER results demonstrated that the overall differences seen in gut microbial community were based on type of diet and feeding period. There was a gradual shift in bacterial communities between fish fed the commercial diet (day 0) and those fed the experimental diets (day 10–30)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



composition was expected to be similar at day 0, since fish in all treatment tanks were fed the same commercial diet during the previous acclimatization period. Within 10 days of experimental diet feeding, microbial composition in the fish gut had changed significantly, indicating that all diets had a significant effect on microbial composition. Previous studies have also reported a change in gut microbiota following a change in diet for salmon, rainbow trout, and brown trout (*Salmo trutta*) after first feeding (Ingerslev et al., 2014b; Michl et al., 2017, 2019). In the studies by Michl and co-workers, trout were fed 0, 50, and 90% plant protein-based diets until 54 days after hatching and then fed a cross-over diet for another 39 days, and a change in microbiome was observed at both 54 and 93 days of feeding showing occurrence of gut microbiota is influenced diet and depend largely on time of sampling. In the present study, diet and 10-day period both had an effect in shaping the gut communities. However, Michl et al. (2017) observed no change in the gut microbiota over time, after a certain point or

with longer feeding duration with the same diet, and concluded that microbiota composition depends largely on the actual diet fed at the time of sample collection. In the present study, the gut microbiota differed significantly at day 10 and day 20, but was similar at day 30 irrespective of different treatment diets, suggesting that obtained microbiota at this time point is not influenced by two of the environmental variations in this case heat processing of diet nor *N. intermedia* inclusion. However, temporal change for all diets from day 20–30 was evident. Until day 20, differences in microbial composition can be in order to adapt to the environment due to dietary intervention. Longer periods of study are needed to confirm this.

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



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

### Effect of Diet and Duration of Feeding on Core Gut Microbiome

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

Data analysis revealed that the next most common phylum in the core gut microbiota after Tenericutes was Firmicutes, followed by Proteobacteria (Figure 1B), as found in other studies on salmonids (Nayak, 2010; Gajardo et al., 2017). One previous study has found that the core gut microbial composition of rainbow trout is resistant to change due to diet type, but that conclusion was reached by comparing data at phylum level (Wong et al., 2013). Another study suggested that there might be differences at lower taxonomic ranks, particularly at species or genus level, rather than at higher taxonomic ranks (Michl et al., 2017). This was the case in the present study, where the abundance of *Lactococcus* (Lactobacillales), *Deefgea* (Neisseriales), and

*Anaerotruncus* (Clostridiales) was significantly enriched from day 0 to day 30 and fish on the preconditioned diet (PD) had higher abundance of *Streptococcus* than those on the non-preconditioned diet (NPD). Lower abundance of bacteria of the genera *Deefgea* and *Anaerotruncus* was observed, as also found in the gut microbiota of humans, rainbow trout, and Atlantic salmon isolated through 16s sequencing (Namsolleck et al., 2004; Perez-Fuentes et al., 2018; Ricaud et al., 2018). *Peptostreptococcus* and *Streptococcus* are generally present in high abundance in protein-rich environments, and play an important role in amino acid catabolism and absorption in the gut (Dai et al., 2011; Davila et al., 2013; Neis et al., 2015). The abundance of one taxa can suppress that of another depending on nutrient availability for growth. A shift in microbial composition from *Streptococcus* to *Lactobacillus* has been reported in Atlantic salmon fed fishmeal-free diets or diets with fishmeal replaced with plant protein (Hartviksen et al., 2014). This is comparable to the results in the present study, where *Peptostreptococcus*, the dominant taxon at day 0 (all fish fed the commercial diet) decreased in abundance with time, whereas abundance of *Lactococcus* increased conferring the change due to the substrate exchange.

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

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

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

## CONCLUSION

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

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the overall gut microbiota continuously changed until day 30, a future research should investigate the further trends of their occurrence.

## DATA AVAILABILITY STATEMENT

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

## ETHICS STATEMENT

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

## AUTHOR CONTRIBUTIONS

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

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## SUPPLEMENTARY MATERIAL

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

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