Sveriges lantbruksuniversitet
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Filamentous fungus *Paecilomyces variotii* in feed for Rainbow trout *(Oncorhynchus mykiss)*

– Assessment of apparent digestibility and early signs of inflammation

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Abstract

Aquaculture production has been increasing rapidly over the last few decades and therefore the amount of feed needed for the increasing production is also on the rise. Salmonids and other farmed carnivorous fish require high amount of protein in their diets, which is currently supplied mostly by fish meal or soy bean. Therefore, there is a need to identify other alternative sustainable protein sources, preferably those that are not grown on arable land or can be used directly for human consumption. Single cell protein are microorganisms such as fungi, bacteria, yeast and mould, that contain a high level of protein and can be grown on various substrates such as residues from other industries. Filamentous fungus *Paecilomyces variotii* is a mould that can be grown on residues from the wood industry and is characterized by a high protein content.

The objective of this thesis is to assess the potential of using *P. variotii* as protein source in diets for rainbow trout (*Onchorhyncus mykiss*) through assessment of apparent digestibility. In addition, intestinal samples were taken for histological analysis of early signs of intestinal inflammation. *P. variotii* has been included in diets to rainbow trout at 20% and 30% inclusion levels. Results show that the inclusion of *P. variotii* at all levels lowered the apparent digestibility coefficient of the diet dry matter, gross energy and crude protein. However, apparent digestibility of the test ingredient was higher for dry matter, energy and crude protein when the test ingredient was exposed to double extrusion during the feed production. Signs of inflamed intestines were least present in fish fed diet with 20% inclusion of test ingredient which may indicate a positive effect of *P. variotii* in the gut at lower inclusion levels.

Keywords: Rainbow trout, single cell protein, *Paecilomyces variotii*, apparent digestibility coefficient, inflammation.

Sammanfattning

Produktionen av akvakultur har hastigt ökat de senaste årtiondena och därmed även mängden foder som behövs inom produktionen. Laxfiskar, och andra fiskar som används inom fiskodlingen och är karnivorer, behöver en viss andel protein i deras foder vilket vanligtvis tillförs av fiskmjöl eller soja för närvarande. Det finns därför ett behov att identifiera alternativa hållbara proteinfoder som kan ersätta fiskmjöl och andra produkter, företrädesvis foder som inte kräver odlingsbar mark eller som kan användas som råvara direkt till människor. Encelligt protein är mikroorganismer som svampar, bakterier, jäst och mögel som innehåller en stor andel protein och kan växa på ett flertal olika substrat, som restprodukter från andra industrier. *Paecilomyces variotii* är en mögelsort som kan växa på restprodukter från skogsindustrin och kännetecknas av ett högt proteininnehåll.

Syftet med denna uppsats är att utvärdera potentialen att använda *P. variotii* som proteinkälla i foder till regnbåge (*Oncorhynchus mykiss*) genom att mäta skenbar smältbarhet. Utöver det, utfördes histologisk analys av tarmprover för att undersöka tidiga tecken på inflammation. *P. variotii* blev inkluderat i dieter till regnbåge med 20% och 30%. Resultatet visar att inkludering av *P. variotii*, både 20% och 30%, minskade koefficienten för skenbar smältbarhet gällande torrsubstans, energi och råprotein. Resultatet för skenbar smältbarhet av testingrediensen var dock högre gällande torrsubstans, energi och råprotein då testingrediensen blivit extruderad vid två tillfällen under fodertillverkningen. Tecken på inflammation i tarmarna var minst hos de fiskar som blivit fodrade med dieten innehållande 20 % av testingrediensen vilket skulle kunna indikera en positiv effekt av *P. variotii* på tarmarna vid en låg inblandning.

Nyckelord: Regnbåge, encelligt protein, *Paecilomyces variotii*, skenbar smältbarhetskoefficient, inflammation.

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1 Introduction

Aquaculture is the fastest growing food producing sector globally and has an important role in providing protein to the global human population (FAO, 2018). Today, almost half of the fish consumed comes from aquaculture (FAO, 2018). In Sweden, total production of food fish in 2017 was at a level of 12 800 ton (slaughtered fresh weight). Rainbow trout (*Oncorhynchus mykiss*) was the most farmed species with production levels of over 11 000 ton (Statistiska Centralbyrån, 2018). Rainbow trout is a salmonid fish species characterized with relatively high requirement for protein in their diet due to carnivorous nature.

The environmental impacts of fish farms are important to consider for the growing production and feed is one of the major environmental impacts from salmonid fish farms according to Life Cycle Analyses (LCA) (Ayer and Tyedmers, 2009; d'Orbcastel et al., 2009; Pelletier et al., 2009). The most commonly protein source used commercially is fish meal. Fishmeal has a high level of protein and the amino acid composition meets the nutrient requirement of most farmed fish. It is however a limited resource and usually made of small species of wild caught fish. Despite a general decrease in the use of fish meal in fish feed diets over the last decades and increased use of plant-based protein, the pressure on wild fish stocks for fish meal production is not decreasing. This is largely due to a rapid growth of aquaculture and increased production levels (Hardy, 2010).

Soybean and its derivatives are widely used as a fishmeal replacement, however soybean as a protein source in aquaculture may not be suitable in the long run as it needs arable land to be produced and it can be used directly for human consumption. In order to keep up with the aquaculture expansion and raising production levels, there is a strong need to search for sustainable protein sources for fish feed that do not compete with human food production.

Sweden has a large forestry industry with residues that can be used for feed production. According to Alriksson *et al.* (2014) by-products from the forestry industry are an interesting alternative for fish feed. Single Cell Protein (SCP) is protein de-

rived from microorganisms such as yeasts, fungi, algae and bacteria. Microorganisms, contain relatively high protein levels, pose no demand on arable land and they can grow on a range of substrates (Nasseri et al., 2011). Restrain regarding SCP is the relatively high content of nucleic acid (NA) which varies from 10 to 15 % (Rivière, 1977 in Nasseri et al., 2011). In brewer's yeast, NA can constitute 12 to 20% of total Nitrogen, mostly in the form of ribonucleic acid (Rumsey et al., 1992). Unlike mammals, who suffer toxicological and metabolic disturbances with high dietary NA concentrations, fish can cope due to high liver uricase activity (Kinsella et al., 1985; Rumsey et al., 1991b).

In order to assess the potential of novel ingredients for their use in fish feed, utilization of nutrients needs to be evaluated. One way and usually the first step in determining the potential of a protein source is to assess the apparent digestibility (Nasseri et al., 2011). Several feeding trials using different sourced SCPs, partly replacing fish meal, have been conducted in the past (Alriksson et al., 2014; Langeland et al., 2016; Øverland et al., 2013; Vidakovic et al., 2016). The study by Øverland *et al.*, (2013) found that moderate levels of yeast *Saccharomyces cerevisiae* affected the growth performance and nutrient utilization negatively for Atlantic salmon (*Salmo salar*), while yeasts *Candida utilis* and *Kluyveromyces marxianus* were shown to be capable of replacing 40% of the fish meal protein without decresing the growth performance, digestibility or nutrient retention. Both Langeland *et al.* (2016), and Vidakovic *et al.* (2016) assessed the apparent digestibility coefficients (ADC) of *S. cerevisiae*, processed in two different ways, and one filamentous fungus *Rhizopus oryzae* in diets for Arctic charr (*Salvelinus alpinus*). In the study by Langeland *et al*. (2016), the authors found that extracted S. cerevisiae had higher ADC for indispensable amino acids than intact *S. cerevisiae* and *R. Oryzae*. However, no difference between extracted *S. cerevisiae* and *R. Oryzae* regarding dry matter, sum of amino acids and energy. In the study by Vidakovic *et al*. (2016), the results indicates that intact *S. cerevisiae* can replace fish meal with 40% on crude protein basis without affecting growth and retention of crude protein and amino acids. These studies and their results for SCP as protein source seems overall promising, however pointing to certain limitations in the use of high levels in fish feeds.

During feed manufacturing, fish feed is formed into pellets, commonly made using extrusion technology, and this process can have impact on the digestibility (Sørensen, 2012). Extrusion is a combination of moisture, pressure, temperature and mechanical shear that Vidakovic *et al*. (2016) found to possibly increase ADC values in their feed trial.

Alriksson *et al*. (2014) used 4 different SCPs in their study, *C. utilis* and filamentous fungi *Fusarium venenatum, R. oryzae*, and *Paecilomyces variotii*. Accord-

ing to the authors, mould *P. variotii* can be used in a diet for Nile Tilapia *(Oreochromis niloticus)* replacing 38% of fishmeal without negative effect on growth. *P. variotii* is a filamentous fungus with cell walls containing chitin, mannoproteins and β-glucans with 1,3- and 1,6-linkage (Brul et al., 1997) and can be grown on residues from the forestry industry (Alriksson et al., 2014). Chitin has been shown to have immunostimulant effects in many fish species when supplemented in feed (Ringø et al., 2012). Similarly, β-glucans have immunostimulant properties in fish, with possible improvement on health and growth (Ganguly et al., 2010). Hence, commercially use of SCP in fish industry has been mostly as probiotics. Products deriving from SCP for rainbow trout can also be used for immune stimulating properties (reviewed by Navarrete and Tovar-Ramrez, 2014). Also, SCPs is used as fish feed additives as aroma and vitamin carriers and as emulsifying aid (Nasseri et al., 2011) but not as main protein source.

Another important aspect to consider when performing nutritional evaluation of novel ingredients to fish is the health status of the fish. A diet with a poor nutritional value or one that contain anti nutritional factors can negatively affect important production parameters as growth rate, but also lead to impaired gut health. Impaired gut health can in turn lead to impaired welfare for the fish (Segner et al., 2012). Morphological changes in intestines can occur due to inflammation which have been studied by Knudsen *et al.* (2008) on Atlantic salmon. The authors found that soy bean saponins can trigger inflammatory response in the distal intestine. Arctic charr fed SCP *S. cerevisiae* and *R. Oryzae* had impaired intestinal barrier function in study by Vidakovic *et al.* (2016) which in turn can lead to an increased risk of evolving inflammation (Segner et al., 2012).

Morphological changes from inflammation in intestine can occur on the epithelial morphology with swollen and shortened folds having increased connective tissue in the base of the folds and lamina propria. Also, number of cells like lymphocytes, goblet cells and eosinophilic granulocytes can increase as an inflammatory response (Knudsen et al., 2008; Uràn et al., 2008).

The objective of this thesis is to evaluate the potential of *P. variotii* as a protein source in diets for rainbow trout (*Oncorhynchus mykiss*) by assessing the apparent digestibility coefficients (ADC) of various nutrients, amino acids and energy. Furthermore, the thesis focuses on evaluating the possible health effects of *P. variotii* in feed on the intestinal health of rainbow trout.

2 Material and methods

2.1 Fish and facilities

Rainbow trout were acquired from Vilstena fiskodling AB (Fjärdhundra, Sweden) and where kept in 500 litre holding tanks prior to the experiment. Five days before the experiment all the fish were sorted according to weight and randomly distributed into 16 experimental tanks, with 14 fish in each tank. One day prior to start of experiment the fish were anesthetized with tricaine methane sulphonate (MS-222, Finquel, Scan Aqua AS, Årnes, Norway), concentration of 75 mg/L^{-1}, weighed (70.34 ± 14.69 g) and placed back into the experimental tanks. MS 222 solutions were buffered with sodium bicarbonate in order to prevent gill damage due to shifting pH values. Feeding with experimental diets started the day after. Time for acclimatization in the experimental tanks before experimental diets was fed, was therefore in a total of 6 days.

The experimental tanks, 200 litres in volume, were supplied with partly recirculated water and addition of fresh water with 1 litre/minute. Water temperature (10.6 ± 0.8°C) and dissolved oxygen (9.98 ± 0.24 mg/l) were recorded every other day (HQ40D Portable Multi Meter, Hach, Loveland, CO, USA). Fish were kept at 12:12 light cycle (from 8.00 am to 20.00 pm).

The trial was carried out in line with laws and regulations overseen by the Swedish Board of Agriculture and approved by the Ethical Committee for Animal Experiments in Uppsala, Sweden (dnr 5.8.18-16347/2017).

2.2 Feed and feeding

Diets were produced by extrusion at Natural Resources Institute Finland (Luke) with a twin-screw extruder (3 mm die, BC-45 model, Clextral, Creusot Loir, France). All diets were extruded at high pressure and at the temperature of 140°C, although

higher pressure and friction were used for the experimental diets then for the reference diet (pressure and friction not recorded). Each dietary treatment was randomly assigned to 4 different tanks. Two of three experimental diets were formulated according to the recommendation by Cho & Slinger (1979) for digestibility trials, where reference diet was mixed 70:30 with the test ingredient. The method by Cho & Slinger (1979) is suggested to mimic the practical feeding conditions. One of the experimental diets was mixed reference diet 80:20 with test ingredient in order to have a lower inclusion level for comparison, since there may be a limiting inclusion level for *P. variotii* in fish feed.

In total, there were four diets produced; reference diet, SCP20, SCP30 and SCP30W where the number represents percentage inclusion of *P. variotii* in the diet (on dry matter (DM) basis). *P. variotii* biomass was produced in 600 litre bioreactors by Domsjö fabriker AB. The substrate used was a by-product, surlut, from textile cellulose production.

Diets SCP30W and SCP30 contained the same inclusion level of *P. variotii* while there was a difference in the dietary production process. The test ingredient used in diet SCP30W has been pre-extruded at 135°C in order to check for potential of prolonged exposure to high pressure and temperature on the apparent digestibility of the test ingredient. The pre-extrusion was performed at Research Institutes of Sweden RISE (Gothenburg), using a single screw extruder (TeachLine E20T, Dr Collin GmbH (Germany)), where *P. variotii* was mixed with the wheat flour to act as a carrier during the pre-extrusion. All diets were iso-energetic. Feed formulation is provided in Table 1. Experimental tanks were equipped with automatic belt feeders (Hølland teknologi, Sandnes, Norway) and feeding took place once a day between 10.50 -12.00am.

Table 1. *Feed formulation, g kg^{-1} (DM).*

Ingredients	Reference diet	SCP20	SCP30, SCP30W
Fishmeal	500.0	400.0	350.0
Soy protein	55.0	44.0	38.5
Wheat meal	150.0	120.0	105.0
Wheat gluten	120.0	96.0	84.0
Fish oil	150.0	120.0	105.0
Vitamin mix	20.0	16.0	14.0
TiO_2	5.0	4.0	3.5
P. variotii	-	200.0	300.0

Titanium dioxide (TiO_2) was included in the diets as a marker for digestibility. Feed rations were calculated using the thermal growth coefficient calculation (TGC)

and the theoretical daily specific growth rate (SGR) of 1%. The temperature used was 11°C. Rations changed with one-week periods.

2.3 Sample collection

Each experimental tank was equipped with a collector for feed waste and faeces (Hølland teknologi, Sandnes, Norway). Faeces collection started after fish had been fed experimental diets for five days, in order to compensate for any possible metabolic effects of new diets on ADC during the initial period. From there on, faeces was collected daily, every morning prior to feeding time (8.00 am) and stored at -20°C until analysis. Feed waste was checked for daily, 30 minutes after feeding ended (12.30 pm).

After 44 days of dietary treatment, all fish were anesthetized in a bath with buffered MS-222 solution (75 mg/L^{-1}) and weighed. Four fish per tank were randomly selected and euthanized with an overdose of MS-222 of 300 mg/L^{-1}. Weights of viscera and liver were recorded for calculations of viscerosomatic index (VSI) and hepatosomatic index (HSI).

2.4 Sampling for histology analysis

A total of 8 fish per dietary treatment (2 fish per tank), were euthanized with an overdose of MS-222 of 300 mg/L^{-1}. Weight of viscera and liver were recorded. The intestine, from posterior to the pyloric caeca to the anus, was dissected and divided into a proximal and distal region at the ilea-rectal valve. Samples of 5 mm, cross section, from the anterior part of each intestinal region were collected.

Samples were washed in deionized water, placed in a cassette and thereafter fixed in containers with 4 % paraformaldehyde in 0.1 M phosphate buffer (room temperature, > 24h). After fixation, the samples where dehydrated and embedded in paraffin wax. Sections of 4 µm were made using a rotary microtome Thermo Scientific Microm HM355S (Thermo Fisher Scientific, Waltham, Massachusetts, USA), before staining with Haematoxylin and Eosin.

2.5 Analyses

Digestibility:

Feed were milled and stored before the analyses. Faeces were freeze dried, weighed, milled and stored before analyses. Determination of DM content was performed by drying the samples at 103°C for 16 hours. Samples were then cooled in a desiccator

and weighed. Determination of ash was performed by incineration at 550°C for 3 hours until the ash was white, sample was then cooled in a desiccator, and weighed. Determination of GE was performed using isoperibol bomb calorimeter (Parr 6300; Parr Instrument Company, Moline, IL, USA) and expressed as MJ kg^{-1}. Total nitrogen determination was performed according to the Kjeldahl method with a digester and analyser (2020 and 2400 Kjeltec; FOSS Analytical A/S, Hilleröd, Denmark). A factor of N x 6.25 was used to determine crude protein (Nordic committee on food analysis, 1976). Crude fat was determined with acid hydrolysis and extraction according to the Official Journal of the European Communities (1998) using a Hydrotec 8000 and a Soxtec 8000 Extraction Unit (FOSS Analytical A/S, Hilleröd, Denmark). Amino acid content in feed and faeces were analysed with SS-EN ISO-13903 (2005) method by Eurofins Food & Feed Testing Sweden (Linköping). Neutral detergent fibre (NDF) was analysed using a 100% neutral detergent solution, while amylase and sulphite were used for the reduction of starch and protein, according to Chai & Udén (1998).

Histology:

Samples, one section from proximal intestine and one section from distal intestine per fish, were blindly evaluated in a Nikon eclipse E600 microscope with a Nikon digital camera Dxm1200 and software Nikon ACT-1 (Nikon corporation, Tokyo, Japan). The intestinal samples were analysed by a scoring system based on Knudsen *et al*. (2008) and Uràn *et al*. (2008), both used for examination of enteritis in Atlantic salmon, but with slight modifications. See table 2. for scoring of histological analyses.

Scoring of following morphology was made from 1-5, where 1 was considered no inflammation and 5 severe inflammation:

Connective tissue: Distance from stratum compactum to the base of the folds. Thin or absent connective tissue indicated a normal intestine. Thick connective tissue indicated an inflammation.

Lamina propria (LP) of simple- and complex fold (complex fold only in distal intestine): Thin LP indicated a normal intestine whereas a swollen LP indicated inflammation. Attachment of the lamina propria to the epithelial cells was considered normal while separated indicated inflammation.

Mucosal folds: Long and thin folds were considered normal. Short and thick folds were assessed as inflammation.

Vacuoles (only distal intestine): Large and many vacuoles filling the epithelial cells indicated normal intestine while small and few vacuoles represent inflammation.

Following morphology was evaluated as normal or deviant from normal:

Eosinophilic granulocytes: Thin layer in the stratum granulosum was considered normal, also appearance of some single eosinophilic granulocytes in folds. Other findings were interpreted as abnormal.

Goblet cells (only proximal intestine): Number and distribution where accumulations of goblet cells were interpreted as abnormal.

Lymphocytes: appearance in LP and among the epithelial cells were considered normal if spread and in a relatively low amount whereas accumulations and increase in numbers were considered as signs of inflammation.

Submucosa: checked for deviation in colour, form, thickness.

After evaluation with the scoring system, signs of inflammation were recorded with yes or no.

Table 2. *Scoring for histological analyses, high values represent sings of inflammation.*

	Proximal	Distal
Connective tissue	1-5	1-5
Eosinophilic granulocytes[1]	Normal or deviant	Normal or deviant
Goblet cells[2]	Normal or deviant	-
Lamina propria, simple folds	1-5	1-5
Lamina propria, complex folds	-	1-5
Lymphocytes[1]	Normal or deviant	Normal or deviant
Mucosal folds	1-5	1-5
Submucosa[3]	Normal or deviant	Normal or deviant
Vacuoles	-	1-5

[1] Location and quantity

[2] Number and spreading

[3] Colour, form, thickness

2.6 Calculations

Calculations were made for TGC, weight gain, HSI, VSI, ADC of diet and ADC of test ingredient according to following equations:

TGC according to National Research Council (U.S.) (2011):

$$TGC = (FBW^{1/3} - IBW^{1/3}) / \Sigma (T \times D) \times 100$$

$$\text{Predicted final body weight (FBW) (g/fish)} = (IBW^{1/3} + \Sigma (TGC/100 \times T \times D))^3$$

IBW= initial body weight (g/fish)
D = number of days
T = water temperature (°C)

Weight gain (%):

Weight gain = ((final weight – initial weight) / initial weight) × 100

HSI and VSI (%):

$HSI = (W_{Liv} / FW) \times 100$

$VSI = (W_{Vis} / FW) \times 100$

ADC of diet (%) according to Cho *et al*. (1982):

$ADC_{Diet} = (1 – (\%\ \text{nutrient in faeces} / \%\ \text{nutrient in feed} \times TiO_2\ \text{in feed} / TiO_2\ \text{in faeces})) \times 100$

ADC of test ingredient in SCP20 (%) according to and modified from Bureau *et al*. (1999):

$ADC_{Test\ ingredient} = ADC_{Test\ diet} + ((ADC_{Test\ diet} – ADC_{Reference\ diet}) \times ((0.8 \times \%\ \text{in reference diet}) / (0.2 \times \%\ \text{in test diet})))$

ADC of test ingredient in SCP30 and SCP30W (%) according to Bureau *et al*. (1999):

$ADC_{Test\ ingredient} = ADC_{Test\ diet} + ((ADC_{Test\ diet} – ADC_{Reference\ diet}) \times ((0.7 \times \%\ \text{in reference diet}) / (0.3 \times \%\ \text{in test diet})))$

2.7 Statistical analyses

The statistical analysis was performed using Graphpad prism 7.04, for windows (GraphPad Software, La Jolla, CA, USA).

The effect of dietary treatment on apparent digestibility coefficients and growth performance was evaluated using one-way ANOVA followed by Tukey's multiple comparisons test. The model included a fixed factor of experimental diet and tank was used as experimental unit. The level of significance was set to $P < 0.05$.

3 Results

3.1 Chemical composition of the test ingredient, feed and faeces

Fish meal contains a higher amount of CP, sum of AA (amino acids) and ash than *P. variotii* whereas *P. variotii* had a higher amount of NDF. *P. variotii* contains all the indispensable amino acids (tryptophan not analysed) though in less amount. Chemical composition of fish meal and *P. variotii* is shown in Table 3.

Table 3. *Proximate composition of fishmeal and P. variotii expressed as g kg^{-1} DM.*

	FM	*P. variotii*
DM (%)	91.0	93.1
CP	762.7	466.2
CF	100.5	86.7
NDF	37.5	71.0
Ash	148.0	47.3
GE (MJ kg^{-1} DM)	22.12	22.07
Sum of AA	594.2	327.8
Indispensable amino acids		
Arginine	36.4	24.4
Histidine	12.7	8.69
Isoleucine	25.6	16.0
Leucine	49.3	27.0
Lysine	49.6	24.6
Methionine	18.7	6.16
Phenylalanine	25.6	15.3
Threonine	28.1	17.3
Valine	31.3	19.4
Dispensable amino acids		
Alanine	39.3	23.0
Aspartic acid	60.3	33.5
Cysteine	5.59	3.46
Glutamic acid	90.8	46.2
Glycine	39.1	17.2
Hydroxyproline	4.32	<0.05[a]
Ornithine	<0.01[a]	0.61
Proline	27.0	17.2
Serine	3.31	1.99
Tyrosine	23.0	14.3

[a] as is, g 100g^{-1}

Chemical composition of the reference diet had a higher amount of CP, sum of AA and ash whereas experimental diets contained more NDF. Chemical composition of diets is shown in Table 4.

Table 4. *Chemical composition of experimental diets expressed as g kg^{-1} DM.*

	Refer-ence diet	SCP20	SCP30	SCP30W
DM (%)	96.4	95.2	95.7	95.0
CP	549.8	529.4	519.3	495.6
CF	161.1	184.3	167.0	162.7
NDF	45.6	65.1	66.9	52.6
Ash	89.3	77.7	74.3	69.0
GE (MJ kg^{-1} DM)	23.98	23.50	23.51	23.26
Sum of AA	478.5	440.2	431.2	404.8
Indispensable amino acids				
Arginine	27.6	26.0	26.6	24.3
Histidine	10.4	10.0	9.86	9.06
Isoleucine	20.0	18.7	18.8	17.5
Leucine	37.9	34.9	34.6	32.3
Lysine	31.2	28.9	28.7	26.3
Methionine	12.9	11.2	10.8	10.2
Phenylalanine	22.0	20.2	19.7	18.6
Threonine	19.9	19.1	19.0	17.8
Valine	23.9	22.7	22.8	20.9
Dispensable amino acids				
Alanine	26.4	25.2	25.6	23.4
Aspartic acid	42.0	39.8	39.2	36.2
Cysteine	5.90	5.35	5.12	4.69
Glutamic acid	98.3	86.4	82.2	79.1
Glycine	27.1	24.7	24.1	22.6
Hydroxyproline	2.00	1.46	1.38	1.40
Ornithine[1]	<0.01	<0.01	<0.01	<0.01
Proline	30.6	26.8	25.3	25.6
Serine	22.2	21.2	20.6	19.7
Tyrosine	18.3	17.5	16.8	15.2

[1] As is, g 100g^{-1}

3.2 ADC

The highest ADC values for DM, CP and GE were observed for the reference followed by SCP20 and SCP30W diet. Lowest values were found for the SCP30 diet. Differences were also present in ADC of amino acids. Values for ADC sum of AA

were highest for reference and SCP20 diet. Similarly, for 6 out of 9 analysed indispensable amino acids; arginine, histidine, isoleucine, leucine, lysine and phenylalanine, ADC was highest for reference and SCP20 diet. Further, SCP30W diet showed no significant difference regarding ADC for arginine and lysine compared to reference and SCP20 diet. Lowest ADC values for amino acids were generally observed for the SCP30 diet although these results were not significantly different from the SCP30W diet. Two amino acids, methionine and valine, did not differ in ADC between any of the diets. The values for ADC of diets are presented in table 5. No feed waste was observed for any dietary treatment during the experiment.

Table 5. *Apparent Digestibility Coefficients of diets (% DM).*

	Reference diet	SCP20	SCP30	SCP30W	*P*-value
DM	87.5[a]	81.4[b]	77.0[c]	81.2[b]	<0.0001
CP	94.4[a]	92.8[b]	91.2[c]	92.8[b]	<0.0001
CF	94.2	94.9	90.2	93.6	0.1019
GE	91.5[a]	86.1[b]	82.0[c]	85.7[b]	<0.0001
Sum of AA[1,2]	96.6[a]	95.7[ab]	94.2[c]	95.4[bc]	0.0013
Indispensable amino acids[1]					
Arginine	97.0[a]	96.1[ab]	95.5[b]	96.0[ab]	0.0191
Histidine	96.5[a]	95.3[ab]	93.4[c]	94.7[bc]	0.0008
Isoleucine	96.2[a]	94.9[ab]	93.4[b]	94.6[b]	0.0023
Leucine	96.7[a]	95.5[ab]	94.1[b]	95.2[b]	0.0017
Lysine	97.5[a]	96.6[ab]	95.3[b]	96.3[ab]	0.0037
Methionine	96.1	95.6	94.0	94.5	0.0725
Phenylalanine	96.0[a]	95.0[ab]	94.0[b]	94.7[b]	0.0028
Threonine	95.8[a]	94.1[b]	92.3[b]	93.8[b]	0.0008
Valine	96.2	95.0	93.4	96.1	0.2211

a,b,c within rows corresponds to significant difference ($P < 0.05$)

n=4, [1] Reference n=4, SCP20 n=2, SCP30 n=2, SCP30W n=3.

[2] Hydroxyproline and ornithine not included

For *P. variotii*, significantly higher values were observed for ADC of test ingredient in SCP30W when compared to SCP30 diet for DM, CP and GE. No differences were observed between the SCP20 and SCP30 diet. Furthermore, no differences in ADC of test ingredient were found for the sum of AA or indispensable amino acids between the experimental diets. The values for ADC of *P. variotii* are presented in table 6.

Table 6. *Apparent Digestibility Coefficients of P. variotii (% DM).*

	SCP20	SCP30	SCP30W	*P*-value
DM	55.9[ab]	51.5[a]	66.0[b]	0.0090
CP	85.3[ab]	82.6[a]	88.4[b]	0.0133
CF	99.8	72.8	90.9	0.1170
GE	62.5[ab]	57.8[a]	70.9[b]	0.0179
Sum of AA[1,2]	90.8	86.3	91.7	0.1194
Indispensable amino acids[1]				
Arginine	92.1	91.9	93.5	0.6128
Histidine	90.4	85.3	90.1	0.1939
Isoleucine	88.8	85.8	90.3	0.2495
Leucine	89.4	86.4	90.7	0.2227
Lysine	92.2	89.4	93.2	0.2345
Methionine	91.5	84.3	87.4	0.4931
Phenylalanine	89.4	87.6	90.6	0.3378
Threonine	86.8	83.6	88.8	0.1553
Valine	89.4	86.0	95.7	0.3803

a,b,c within rows corresponds to significant difference ($P < 0.05$)

n=4, [1] Reference n=4, SCP20 n=2, SCP30 n=2, SCP30W n=3.

[2] Hydroxyproline and ornithine not included

3.3 Histology

Fish showing signs of inflammation from different diets are presented in table 7. Histological analysis showed no signs of inflammation in the fish fed SCP20 diet. Fish fed SCP30 diet displayed most signs of inflammation followed by the fish fed reference diet. No fish showed signs of inflammation in both proximal and distal intestine, but only one of these regions. All fish with signs of inflammation in proximal intestines were evaluated to be mild. One of the fish fed reference diet showed mild signs of inflammation in the distal intestine and one severe signs of inflammation in the distal intestine, the same for SCP30W diet. For SCP30 diet, two fish showed mild signs of inflammation in the distal intestine and one severe signs of inflammation in the distal intestine.

Table 7. *Number of fish per diet with signs of inflammation in the intestines, n=8.*

	Reference diet	SCP20	SCP30	SCP30W
Proximal	1	0	3	0
Distal	2	0	3	2

Pictures with example of proximate intestines evaluated normal and suspected inflamed are shown in figure 1 and figure 2. Pictures with example of distal intestine evaluated normal and suspected inflamed are shown in figure 3 and figure 4.

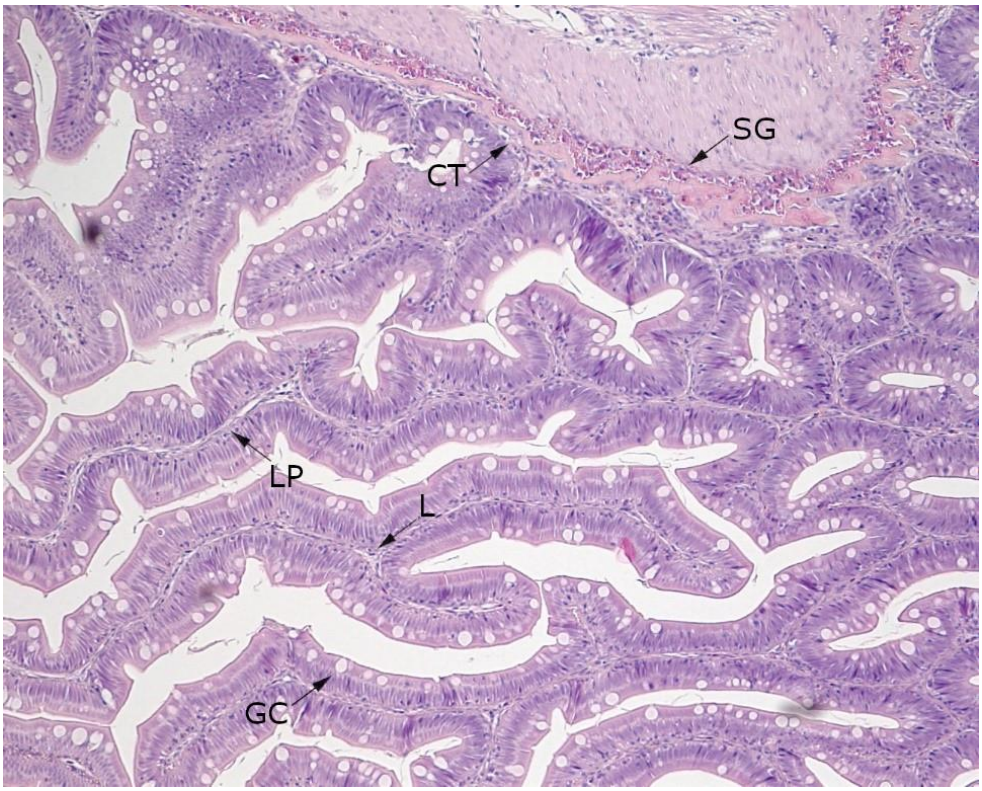

Figure 1. Proximal intestine evaluated as normal.

Figure 1. shows a generally thin connective tissue (CT) in bases of folds. Stratum granulosum (SG) containing eosinophilic granulocytes (EGC) is also generally thin and EGC does not appear in access in folds. Goblet cells (GC) and lymphocytes (L) not deviant from normal. Mucosal folds are thin and long with a thin lamina propria (LP) attached to the epithelial cells.

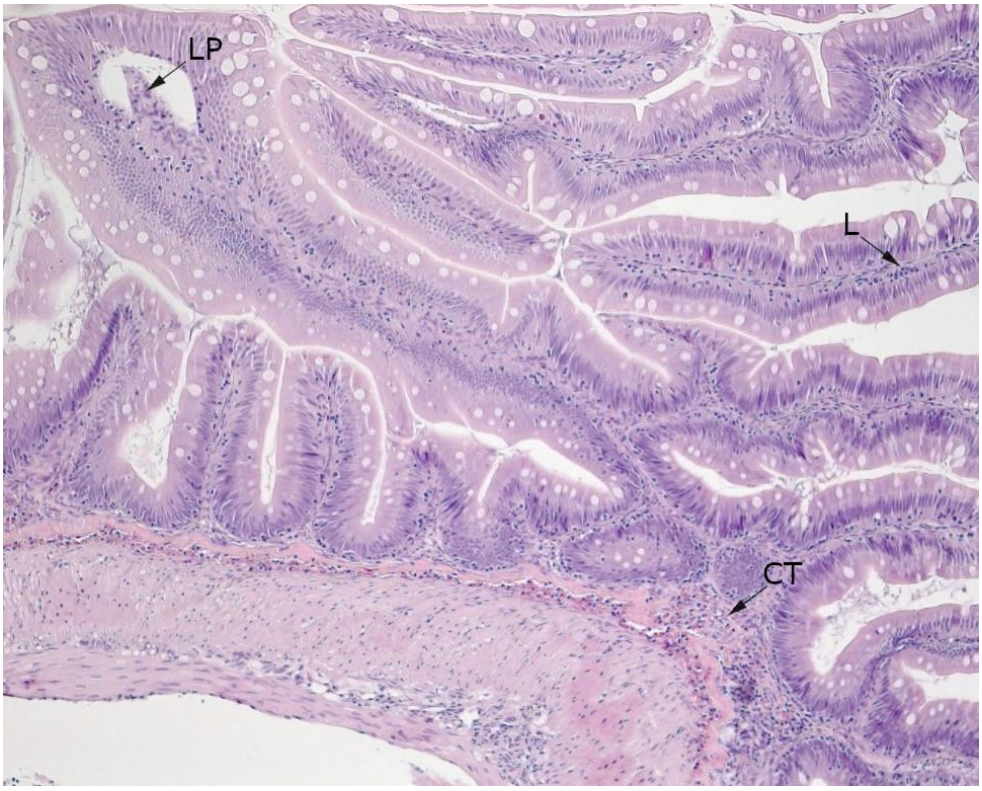

Figure 2. Proximal intestine evaluated as potentially inflamed.

Figure 2. shows a thin connective tissue (CT) in some bases of the folds but a heightening under others. Mucosal folds are swollen, and some are short and stubby. The lamina propria (LP) loses attachment to the epithelial cells and contains lymphocytes, more than normal.

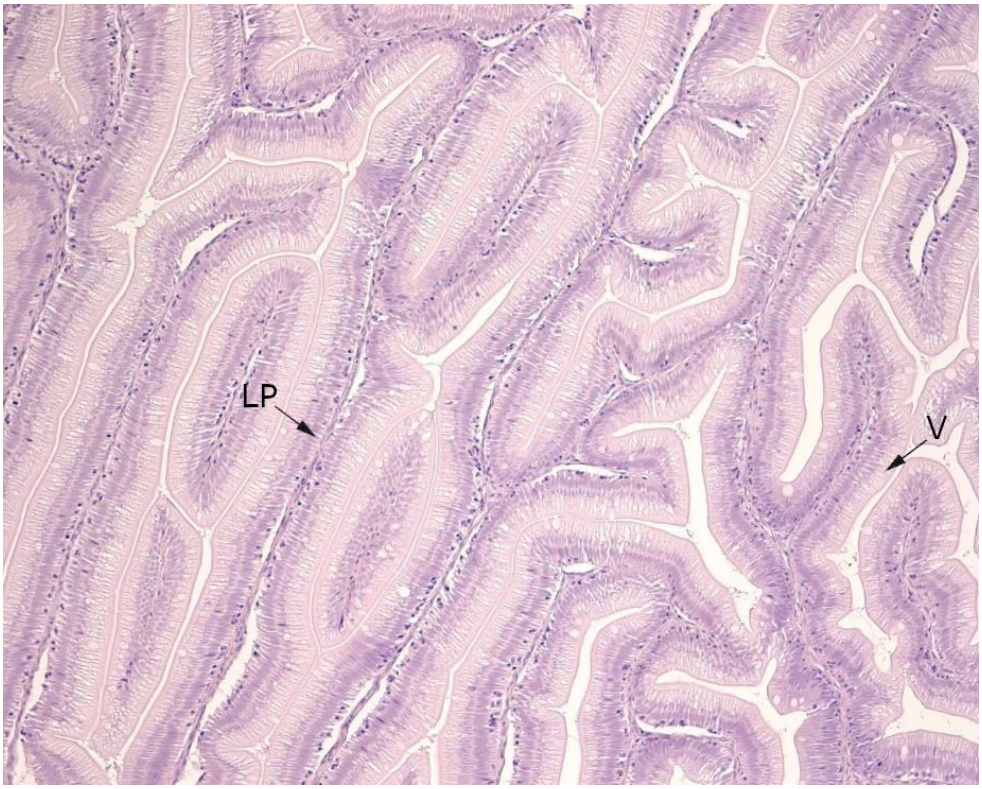

Figure 3. Distal intestine evaluated as normal.

Figure 3. shows long and thin mucosal folds. The lamina propria (LP) is also thin. Vacuoles (V) are large and many, filling the epithelial cells.

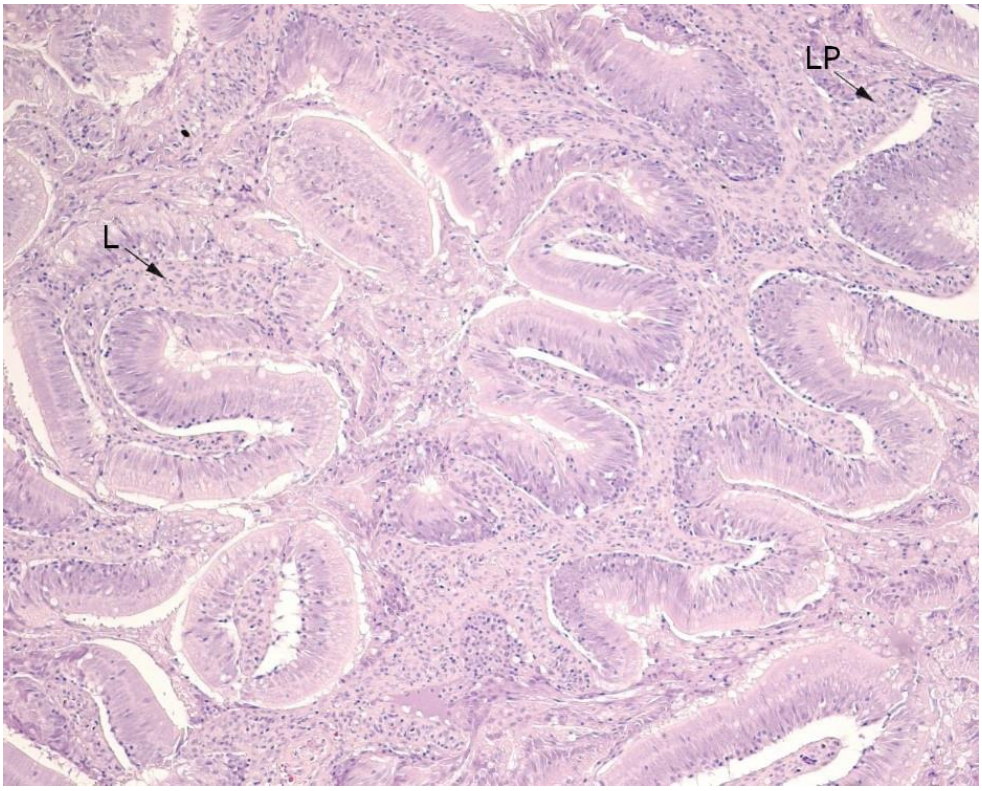

Figure 4. Distal intestine evaluated as inflamed.

Figure 4. shows swollen, short and stubby mucosal folds with an excessive number of lymphocytes in the lamina propria (LP). The LP has also lost attachment to epithelial cells and the vacuoles is not prominent.

3.4 Growth and relative body indices

Weight at start did not differ between any of the groups. Fish fed diets including *P. variotii*, in all levels, had significantly lower weight gain compared to the fish fed the reference diet ($P = 0.0003$). There were no differences regarding VSI between the groups. However, fish fed the SCP30W diet had higher HSI in comparison to the fish fed the other diets. Growth and relative body indices are shown in table 8.

Table 8. *Growth and relative body indices, average ± SEM.*

	Reference diet	SCP20	SCP30	SCP30W	SEM	*P*-value
Weight start	70.13	71.85	70.33	69.03	0.580	0.5938
Weight end	134.5	129.7	126.5	125.3	2.055	0.1821
Weight gain	91.73[a]	80.53[b]	79.85[b]	81.53[b]	2.795	0.0003
HSI	1.319[a]	1.288[a]	1.262[a]	1.522[b]	0.066	0.0011
VSI	9.549	8.786	9.751	9.442	0.209	0.3310
Mortality	1.79%	0	0	1.79%	-	-

Weight start n=56

Weight end, reference and SCP30W n=55. Weight end, SCP20 and SCP30 n=56

HSI and VSI, n=16

4 Discussion

The result shows that the apparent digestibility values for DM, CP and GE were significantly higher for reference diet than for any of the experimental diets. The chemical composition of the fish meal shows higher CP content than the test ingredient, and likewise the chemical composition of the reference diet shows higher values regarding CP than the experimental diets.

In addition to a lower CP content in all experimental diets, SCPs may contain nucleic acid (NA). Since CP content in feed and faeces is based on total amount of nitrogen, it is possible that some of the CP was in fact non-protein nitrogen (NPN) in the form of NA. However, suggestion has been made by Rumsey *et al.* (1992) that rainbow trout can use NPN and NA for dispensable amino acid biosynthesis. Furthermore, nucleotides in diet can function as palatability enhancer in feed, increase growth and modulate the immune response of the fish (Li and Gatlin, 2006).

Arctic charr (*Salvelinus alpinus*) fed extracted yeast *Saccharomyces cerevisiae* or Zygomycetes *Rhizopus oryzae* in diets, inclusion 30%, effected ADC positively for DM and extracted *S. cerevisiae* led to higher ADC value for GE (Langeland et al., 2016). In another study by Vidakovic *et al*. (2016), replacing 40% of the fish meal with the same test ingredients as the study above and on the same species, showed lower ADC for DM when feeding *R. oryzae*. Also, *R. oryzae* and intact *S. cerevisiae* had lower ADC for CP. Further, Øverland et al. (2013) reported that replacement of 30% fishmeal with yeast *Candida utilis* and *Kluyveromyces marxianus* in diets for Atlantic salmon (*Salmo salar*) did not affect ADC for CP or energy. However, inclusion of *S. cerevisiae* gave lower ADC for CP and energy in comparison to the other diets. In a study with yeast in feed for Rainbow trout, the authors found that the cell wall of *S. cerevisiae*, if intact, can lead to lowered bioavailability of nutrients (Rumsey et al.,1991a). Both cell walls of yeast and filamentous fungi contains mannoprotein, β-glucans and chitin yet more chitin in most filamentous fungi (Brul et al., 1997).

Some of the results above are consistent with results in this study, that inclusion of SCP can lead to a lowered ADC for DM, CP and GE. There are however differences between studies that can be due to different SCP having different chemical composition (Nasseri et al., 2011). In addition, the same species of SCP can have differences in nutritional quality depending on growth substrate and conditions. Further, the feed manufacturing can affect digestibility depending on production process, for example differences in temperatures and pressure. Also, the method for collecting faeces and choice of digestibility marker can affect ADC, where some methods tends to overestimate whereas others tends to underestimate (Hajen et al., 1993; Vandenberg and De La Noue, 2001).

Values for ADC of sum of amino acids did not differ between reference diet and SCP20 diet, which also was the case for most of the indispensable amino acids. Threonine was the only amino acid showing significantly higher ADC value in reference diet than in any of experimental diets. Arginine and lysine showed no significant difference in ADC values between reference diet, SCP20 and SCP30W, but reference diet and SCP30 diet differed, the latter having the lowest values. There was no difference in ADC values for methionine and valine between any of the diets. Compared to other literature (Langeland et al., 2016; Øverland et al., 2013; Vidakovic et al., 2016) the general ADC of the amino acids was very high. Furthermore, limiting amino acids is lysine and methionine (National Research Council (U.S.), 2011). Methionine had no difference in ADC between diets, and lysine only significantly lower ADC for SPC30 diet. For the test ingredient, there was no significant difference in ADC for amino acids between any diet. Further results for *P. variotii* show that ADC values for SCP30W was significantly higher for DM, CP and GE in comparison to SCP30, but not when lower inclusion of test ingredient in SCP20. Since the difference in producing SCP30 and SCP30W was the additional pre-extrusion made on the *P. variotii* together with wheat flour in the SCP30W diet, the pre-extrusion may have increased the ADC of *P. variotii* through additional heat, pressure and shear. Effect of extrusion on ADC of GE has earlier been observed by Glencross *et al*. (2011) in a study on grains for rainbow trout. In that study though, there was no indication of positive effects on digestibility of protein. Extrusion of *S. cerevisiae* in feed for Arctic charr has previously been suggested to increase digestibility by disruption of cell walls (Vidakovic et al., 2016). When cell walls have been disrupted, intracellular protein, and other nutrients, can be released and more easily digested by salmonids (Rumsey et al., 1991a; Rumsey and Hughes, 1990). In a study feeding solvent extracted soybean meal to rainbow trout, the authors precooked the test ingredient before extrusion. The pre-cooking led to increased values for ADC regarding carbohydrates, energy and organic matter compared to the extrusion of raw materials (Barrows et al., 2007).

Accordingly, for this thesis, ADC of *P. variotii* for DM and GE showed differences between pre-extruded and not, but no difference regarding any individual indispensable amino acids. It may though be possible that the pre-extrusion led to a higher level of disruption of the cell walls releasing more intracellular protein which was more easily digested by the fish, and therefore the higher value of ADC for CP.

The histology analysis show that fish fed SCP20 diet had least signs of inflamed intestines, even in comparison to fish fed reference diet. Although the cause for such results remains unclear, it could be speculated that 20% inclusion of *P. variotii* might be beneficial for the intestinal health in rainbow trout. However, this speculated potential effect did not reflect on digestibility or growth performance during the experiment.

Chitin can affect performance of fish differently depending on if the chitin is being absorbed in the gastro-intestinal tract. If the fish can utilise chitin it may contribute to positive effects on the gut bacteria, function as immunostimulant, protect the fish from pathogens and therefore increase the overall welfare for the fish, whereas if not, it can lead to reduced growth rates (Ringø et al., 2012). Differences in utilization of chitin has been recorded in rainbow trout at different stages of life cycle, with juveniles having higher ability to utilize chitin than adults (reviewed by Ringø et al., 2012).

Diets including *P. variotii* also provided β-glucans in the diet, which could have contributed to the fish fed SCP20 diet having less signs of inflamed intestines. However, overdose of immunostimulants can induce immunosuppression (Ganguly et al., 2010) and Ringø *et al.* (2012) suggest that there can be a limiting level of inclusion of chitin where the negative effects balance out the positive effects.

In total 6 fish fed SCP30 diet showed signs of inflammation in proximal or distal intestines, indicating that 30% inclusion of *P. variotii* may be too high. In group fed SCP30W diet, 2 fish showed signs of inflammation in distal intestines, indicating that maybe signs of inflammation can be less if pre-extrusion of *P. variotii* is being conducted when included at level of 30%. The reason behind this suggestion can once again be due to the breakage of cell walls that could have occurred during the pre-extrusion, leading to more easily digested components.

However, due to limited number of fish analysed for intestinal health, further, more comprehensive studies are needed to confirm these findings. In addition, both articles (Knudsen et al., 2008; Uràn et al., 2008) used for formation of scoring protocol were evaluating signs of inflammation in Atlantic salmons fed soy bean derivates that may induce other signs of inflammation than *P. variotii*. There can also be a species difference and a variation in the level of tolerance between individuals.

Fish fed reference diet had higher weight gain when compared to fish fed experimental diets. These differences could be due to a lower content of CP in the experimental diets, where the lower content of CP was more pronounced for the SCP30 and SCP30W diet than for the SCP20 diet. In addition, diets in this experiment were formulated according to the standards by Cho & Slinger (1979) for performing digestibility trials, since the object of this study was digestibility, and were not tailored to suit the nutrient requirement of rainbow trout.

In the study by Alriksson *et al*. (2014), the growth of Tilapia was not negatively affected by inclusion of *P. variotii* in feed. The difference in results with this thesis can be due to several reasons. One obvious difference is the species, Tilapia is an omnivorous fish, differing from rainbow trout regarding anatomy and physiology. There can also be differences of *P. variotii* depending on growth substrates (Nasseri et al., 2011) leading to different quality of the feed. It can also be that diets in this thesis were not balanced to correspond to nutrient requirements, and that diets used in Alriksson *et al.* (2014) was.

Weight gain of Artctic charr fed extracted *S. cerevisiae* and *R. oryzae* was lower than charr fed reference diet with fishmeal, even though extracted *S. cerevisiae* showed highest value of ADC for CP and indispensable amino acids in Vidakovic *et al*. (2016). In the study by Øverland *et al.* (2013) there was no difference regarding weight gain of Atlantic salmon, fed two of three yeast strains (*C. utilis* and *K. marxianus*) in comparison to fish meal group. The lower weight gain of fish fed yeast *S. cerevisiae* is suggested by the authors to be due to lower digestibility of CP, amino acids and GE. That is in accordance with this thesis where all experimental diets had lower values of ADC for CP and GE than reference diet. However, ADC of SCP20 diet showed no significant difference to the reference diet according sum of amino acids, nor most of the indispensable amino acids.

No difference in weight gain was recorded between the SCP30 and SCP30W diet neither. That is in accordance with the study of Barrows *et al*. (2007) where the authors found that pre-cooking test ingredient increased ADC values, but the results did not reflect on the weight gain of the fish.

In terms of relative body indices, fish fed SCP30W diet had higher HSI compared to fish fed the other diets. This could be due to higher degree of starch degradation in the extrusion process, suggesting that the pre-extrusion of *P. variotii* together with the wheat flour, led to higher degree of gelatinization. As it is well established, starch at high temperature and pressure is cooked and broken down into amylase during extrusion and research has shown increased digestibility of starch once it was extruded to rainbow trout (Glencross et al., 2011). In Kim & Kaushik

(1992), they report abnormal enlargement of liver in rainbow trout and that the enlargement could have been due to increased levels of hepatic glycogen deriving from increasing levels of starch.

Øverland *et al*. (2013) also reported larger livers in fish fed *C. utilis* or *K. marxianus* compared to the fishmeal diet, although now clear reasoning for this was implied. One possible explanation is that the pre-extrusion may have caused more carbohydrate compounds from the cell walls to be digested, as a result of possible cell wall breakage, which may indirectly increase the liver size, as dietary carbohydrates for rainbow trout previously has been shown to be positive related to hepatic glycogen and HSI (Kim and Kaushik, 1992). Regarding the results for HSI in this thesis, a longer trial with balanced diets may show if any effects on liver is to be expected due to feed processing.

5 Conclusions

Based on the results of this thesis, highest values for ADC of the experimental diets were observed for the diet with 20% inclusion of *P. variotii* followed by diet with inclusion of 30% pre-extruded *P. variotii*. There was no difference observed for ADC of test ingredient regarding amino acids between the diets. Inclusion of 30% *P. variotii* leads to signs of inflamed intestines and lower ADC. However, pre-extrusion seems to increase the digestibility of *P. variotii* which indicates positive effects of extrusion on digestibility. Based on the histology analysis, inclusion of 20% *P. variotii* may have positive effect on the gut health. All levels of inclusion of *P. variotii* affected growth performance, although caution should be used when interpreting growth performance, since the diets were not formulated according to the nutrient requirements of rainbow trout. Future research needs to be conducted to explore the possibilities and restrains of feeding rainbow trout *P. variotii*, especially in regard to exploring the effects on growth performance.

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Appendix 1

Populärvetenskaplig sammanfattning - Mögel som foder till fisk