Fungal and Mussel Protein Sources in Fish Feed:

Nutritional and Physiological aspects

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Abstract

Fungal and mussel protein sources in the diet of Eurasian perch (*Perca fluviatilis*), Arctic charr (*Salvelinus alpinus*) and rainbow trout (*Oncorhynchus mykiss*) and their effect on growth performance, nutrient utilisation, intestinal barrier function and post-prandial profiles of plasma amino acids and whole blood parameters were evaluated in this thesis.

Apparent digestibility of dry matter, sum of amino acids and gross energy in experiment with Arctic charr decreased with dietary inclusion of intact *Saccharomyces cerevisiae*. No significant differences in apparent digestibility between dietary treatments were found in experiment with Eurasian perch. Growth performance, protein retention and total amino acids in Arctic charr were not affected by diets containing intact *S. cerevisiae* and *M. edulis*, demonstrating that 40% of fish meal can be replaced on crude protein basis with these protein sources in Arctic charr diets. Intestinal barrier analysis showed higher apparent permeability for diets with intact *S. cerevisiae* and *R. oryzae* than for a reference diet. Trans-epithelial resistance in the proximal intestine was not affected by dietary treatment.

In rainbow trout, replacing 40% of fish meal with intact *S. cerevisiae* or *Wickerhamomyces anomalus*/*S. cerevisiae* mix had no effect on feed conversion and growth. Apparent digestibility of crude protein was unaffected when 20% of fish meal was replaced with yeast mix and the overall results of this study demonstrated that 40% of fish meal can be replaced on a digestible protein basis with yeast sources without compromising growth performance.

Analysis of post-prandial dynamics of whole blood parameters and plasma free amino acids in dorsal aorta cannulated rainbow trout fed yeast supplemented diets showed significant increase in whole blood pH, TCO_2, HCO_3 and base excess compared with the reference diet, indicating acute metabolic alkalosis in response to differences in mineral content between these diets. Methionine, hydroxy-lysine-2, 3-methyl-histidine and hydroxy-proline concentrations differed significantly between fish fed yeast-based diets and the reference diet, possibly due to differences in dietary crystalline methionine levels and collagen content between yeast and fish meal sources.

Keywords: Arctic charr, Eurasian perch, Rainbow trout, alternative protein sources, dorsal aorta cannulation, growth performance, digestibility.

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Dedication

To my mother, for her endless support….

Mojoj majci, za bezgraničnu podršku….

Contents

List of Publications

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

I Langeland, M., Vidakovic, A., Vielma, J., Lindberg, J.E., Kiessling, A. & Lundh, T. (2014) Digestibility of microbial and mussel meal for Arctic charr (*Salvelinus alpinus*) and Eurasian perch (*Perca fluviatilis*). *Aquaculture Nutrition***,** DOI: 10.1111/anu.

II Vidakovic, A., Langeland, M., Sundh, H., Sundell, K., Olstorpe, M., Vielma, J., Kiessling, A. & Lundh, T. (2015) Evaluation of growth performance and intestinal barrier function in Arctic charr (*Salvelinus alpinus*) fed yeast (*Saccharomyces cerevisiae*), fungi (*Rhizopus oryzae*) and blue mussel (*Mytilus edulis*). *Aquaculture Nutrition*, DOI: 10.1111/anu.12344

III Vidakovic, A., Huyben, D., Nyman, A., Vielma, J., Olstorpe, M., Passoth, V., Lundh, T. & Kiessling, A. (2015) Evaluation of growth, digestibility and phytase activity of rainbow (*Oncorhynchus mykiss*) trout fed graded levels of yeasts *Saccharomyces cerevisiae* and *Wickerhamomyces anomalus*. (Manuscript)

IV Huyben, D., Vidakovic, A., Nyman, A., Langeland, M., Lundh, T. & Kiessling, A. (2015) Effects of dietary yeasts and acute stress on blood parameters of dorsal aorta cannulated rainbow trout (*Oncorhynchus mykiss*). (Manuscript)

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Abbreviations

ADC	Apparent digestibility coefficient
ATP	Adenosine triphosphate
BE	Base excess
CP	Crude protein
DM	Dry matter
FCR	Feed conversion ratio
GE	Gross energy
Hb	Haemoglobin
HCT	Haematocrit
IAA	Indispensable amino acids
Leu	Leucocrit
RBC	Red blood cell count
SGR	Specific growth rate
UPLC	Ultra performance liquid chromatography

1 Background

1.1 Global aquaculture – current status

Total fish production including both capture fisheries and aquaculture on a world scale, accounted for 158 million tons in 2012. Of this total amount, 66.6 million tons (42.2%) were produced by aquaculture (Figure 1). Food fish output from aquaculture has increased eight fold since the 1950s, making aquaculture the fastest growing food industry. In the period between 2000 and 2012, aquaculture expanded at an annual rate of 6.2 % and food fish production from aquaculture will most likely surpass fisheries production in the next decade (FAO, 2014).

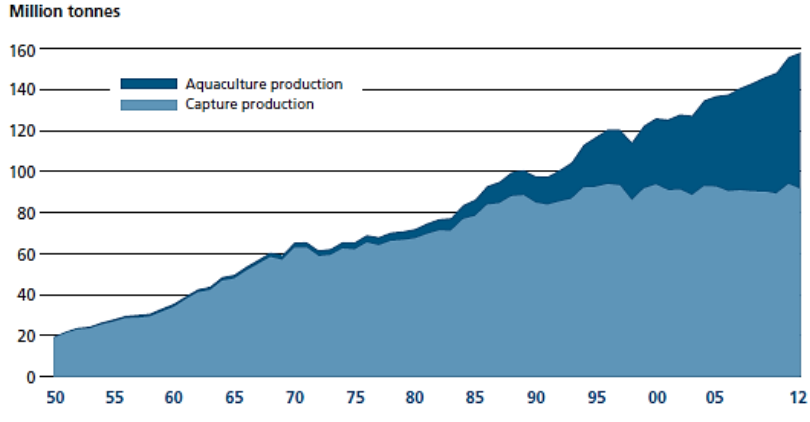

Figure 1. Total world fish production showing the relative contribution of capture fisheries and aquaculture production (FAO, 2014)

In general, 92.7 % of global aquaculture production in 2012 was produced by 15 countries (Figure 2).

At present Asia is leading global production, contributing 88% of total volume produced with the lead producer being China, which farmed over 41 million tons in 2012. However, aquaculture expansion differs greatly between continents. The highest growth rate of aquaculture in the period 2000-2012 was recorded in Africa (11.7 %) while the lowest was in Europe (2.9%). The largest producer in Europe in 2012 was Norway, with 1.3 million tons of fish produced. According to FAO (2014), a total of 354 species of fish were farmed globally in 2012.

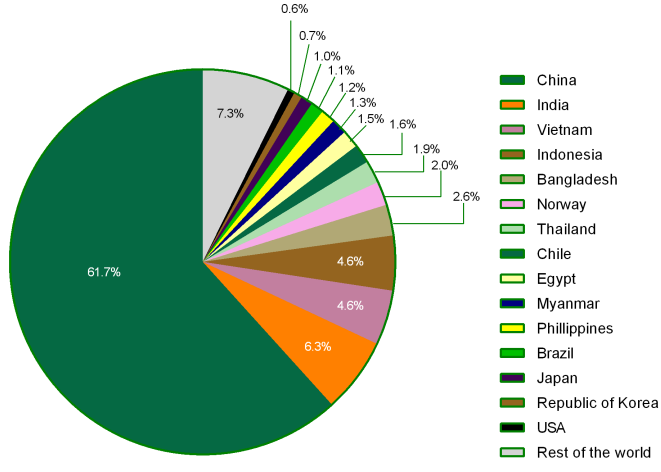

Figure 2. Farmed food fish production by major producing countries in 2012 (FAO, 2014)

1.2 Aquaculture in Sweden

Aquaculture in the Nordic countries has to some extent stagnated over the past decade and it has been suggested that this is due to geographical conditions and somewhat limited natural resources (Dalsgaard *et al.*, 2013). According to Statistics Sweden (2013), total fish production in Sweden in 2013 was 11 663 tons, a 6% decrease compared to 2012. This was the first decline in aquaculture production in recent years. Prior to this decrease, the amount of fish produced in Sweden from 1983 onwards increased steadily over time (Figure 3). A total of 83.6% of all fish farmed in Sweden in 2013 was rainbow trout (*Oncorhynchus mykiss*), while 15.5% of fish produced was Arctic char (*Salvelinus alpinus*). Production of Arctic charr in Sweden has been on the rise in recent years, owing to the favourable growth of this species in the Swedish climate and the existence of a long-term breeding programme (Brännäs & Linnér, 2000).

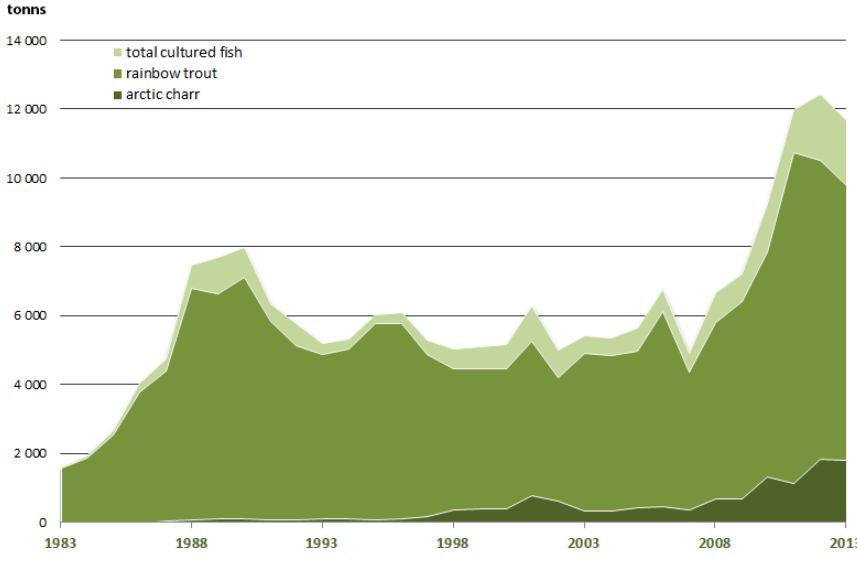

Figure 3. Total fish production and production of rainbow trout and Arctic charr in Sweden (Statistics Sweden, 2013)

1.3 Aquaculture in global food supply

Average world fish consumption is currently at a level of 19.2 kg *per capita* and year. According to FAO (2014), in 2011 nearly one-third of world fish produced was consumed in Asia, where the average annual *per capita* consumption was 21.4 kg. Average fish consumption in 2012 differed between world regions, from 25.1 kg *per capita* and year in Oceania to 9.3 kg *per capita* and year in the Near-East region. Average fish consumption in low-income developing countries has been on the rise over the past few decades and this is being followed by the expansion and growth of aquaculture, although it is still not on a level equivalent to that in developed countries (HLPE, 2014).

Fish supplies approximately 3 billion people with 20 % of their animal protein intake today and another 1.3 billion with 15 % of their protein intake. This includes both captured and farmed fish. As reported by FAO (2012), in some countries, share of protein supply from fish exceeds 50%. In West-African countries such as Ghana and Sierra-Leone, 63% of annual animal protein intake is supplied by fish.

In order to maintain the food supply for a global population which is estimated to reach 9.6 billion people by 2050, agricultural production will have

to increase by 70%. Under current ratios of fish use and general food supply that means a demand for another 47.5 million tons of food fish by 2050 according to estimate by FAO (2014). However this estimate is based on current fish consumption whereas Peterson *et al.* (2007) report that fish food demand in developed countries increases at a rate of 2.5% annually which indicates that demand could be even higher than the current estimate. Aquaculture, with its current growth and potential for expansion could play an important role in supplying high quality protein for the world's population.

1.4 Fish feed, facts and challenges

If aquaculture is to continue its growth at such a high rate, this will have to be matched by equivalent growth in feed supply. At present, the largest operating costs of aquaculture are related to feed and feeding (Tacon & Metian, 2008). One of the main reasons behind such high costs is the cost of protein included in the feed. The feed industry has traditionally used fish meal produced from wild caught fish as the major protein source in aquaculture diets (Gatlin *et al.*, 2007) due to its high nutritional value to farmed fish. Fish meal contains high levels of protein (60-72%) and an amino acid content that covers the indispensable amino acids (IAA) requirements of most farmed fish species. Carnivorous fish species and most marine species tend to have higher dietary requirement for protein than most fresh water species (Wilson, 2002).

Similarly to fish meal, fish oil has traditionally been used as the major lipid source in aquaculture diets. Although the consumption of fish oil by aquaculture has declined somewhat over the past few years, (Shepherd & Jackson, 2013) (Figure 4), mainly due to increased replacement by plant oils, it still remains a major ingredient in diets for certain species, such as salmonids. In 2010, 70% of total fish oil used in aquaculture was used by the salmon industry. The major reason for such high use is the relatively high content of long-chain omega-3 polyunsaturated fatty acids (LC-PUFA) in fish oil, with various documented beneficial effects on human health. When fed to fish, fish oil is transformed into high levels of LC-PUFA in the fillet of salmonid fish, a highly desirable quality trait for Western consumers (NRC, 2011).

It has been proposed recently that the strong expansion of aquaculture may cause the demand to outgrow the supply of fish meal and fish oil in the near future (Naylor *et al.*, 2009; Gatlin *et al.*, 2007). This is supported by recent reports about the global decline in wild catches by FAO (2014) that indicate a possible decline in fish meal and fish oil production in the future. Furthermore, species caught for production of fish meal, which mostly include small pelagic

fish such as anchovies and sardines are increasingly sought for human consumption (Tacon & Metian, 2009).

Nonetheless, the global use of fish meal by the aquaculture industry seems to be on the decline in recent years in actual and relative weight, as predicted by Tacon and Metian (2008). China's aquaculture industry, which has tripled over the past 10 years, today uses only double the amount of fish meal (IFFO, 2015). What is more, salmon feed in 2013, contained as low as 18.3% of fish meal, compared with 65% in 1990. The fish in/fish out ratios (FIFO) in the Norwegian salmon industry has dropped from 4.4 to 1.0, as reported by Ytrestøyl *et al.* (2015). This decrease in the use of fish meal is a result of advances in feed formulation but also an increase in the use of alternative protein sources in an effort by industry to decrease the production costs.

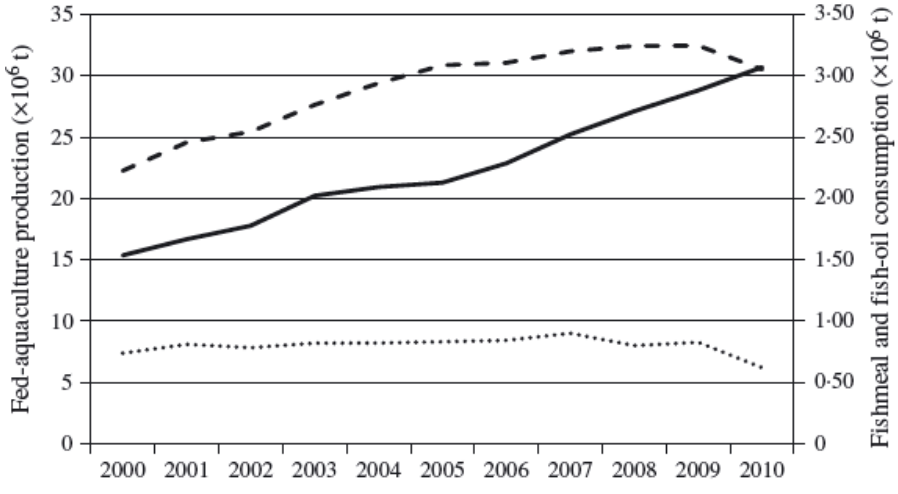

Figure 4. Consumption of fish meal and fish oil and use of fish meal by aquaculture in the period 2000 to 2010. ----, fish meal utilized for aquaculture, —— aquaculture production, ·····, fish oil in aquaculture (Shepherd & Jackson, 2013)

1.5 Alternative protein sources in aquaculture

Growing concern about the sustainability of aquaculture production, economic benefits and limitations in connection with fish meal supply have also triggered an increase in use of fish by-products as a protein source (NRC, 2011). This includes by-products from both aquaculture and capture fisheries and the result is a product similar in quality to fish meal. However, the nutrient sources used as alternatives to fish meal and fish oil are currently mainly plant based (Tacon & Metian, 2008; Gatlin *et al.*, 2007). Fish meal substitutes of plant origin are

primarily legumes or oilseed meals such as soy and sunflower meal. Major disadvantages with the use of these sources are related to presence of anti-nutritional compounds, the need for processing, energy disparity and imbalanced amino acid profile (Krogdahl *et al.*, 2010; Gatlin *et al.*, 2007). In addition, pesticide contamination in plant protein sources could also act as a risk factor.

In some instances, other, animal-based alternatives are used by feed industry to replace the fish meal, mostly in combination with plant protein sources. These include Antarctic krill meal and land animal proteins such as feather meal and meat & bone meal (NRC, 2011). However, krill is costly and a limited resource, while the use of land animal proteins in fish feed is restricted in European Union (FEFAC, 2013) .

Another emerging issue with regard to the use of plants as a fish meal replacement is the requirement for arable land for their production. As the human population grows, there is an increasing need for arable land for production of food for human consumption (Brown, 2012). Producing sufficient food for a growing human population will increase the pressure on natural resources. Therefore, managing and governing these resources for the benefit of food security in the future while meeting these demands is of high importance (HLPE, 2014).

Hence, future alternatives to fish meal should not compete with human food sources and should be produced by other means. Farmed animals, in this case fish, should convert 'non-human' or 'low-interest human' food resources into food suitable for humans in an environmentally friendly manner (Kiessling, 2009).

1.5.1 Microbial protein sources

Usually referred to as 'single-cell proteins', microbial protein sources include either whole organisms or extracts from micro-algae, fungi and bacteria. These microorganisms, if used in aquaculture diets, would not compete with human food sources and could in the long run improve feed availability, a necessary step for expansion of the aquaculture industry (Øverland *et al.*, 2013; Edebo, 2008; Kiessling & Askbrandt, 1993).

Yeasts, which belong to the kingdom of fungi, are not suitable for direct human consumption, as they usually contain elevated levels of nucleic acids (NA) which in the long term can cause an increase in plasma uric acid, leading to gout and formation of kidney stones in mammals (Rumsey *et al.*, 1992). Due to highly active liver uricase, fish such as salmonids can metabolise NA without apparent health impairments (Andersen *et al.*, 2006; Kinsella *et al.*, 1985). Furthermore, yeast derived nucleic acids and cell wall components such

as mannan oligosaccharides (MOS) and β-glucans are used as immuno-stimulants in diets for fish as they have been shown to promote non-specific immune response (Refstie *et al.*, 2010; Gatesoupe, 2007; Paulsen *et al.*, 2003; Oliva-Teles & Gonçalves, 2001). Among the possible benefits of using yeasts and other fungi in diets for fish are relatively high protein content and favourable amino acid profiles (Nasseri *et al.*, 2011). Furthermore, yeasts and other fungi have the ability to utilize various substrates and produce high quantities of protein over short periods (Nasseri *et al.*, 2011; Kiessling, 2009).

Baker's yeast *Saccharomyces cerevisiae* is a well-known and easily available yeast species with a wide range of practical applications. Various strains of yeast *S. cerevisiae* have been used as immunostimulants in previous research with fish (Gatesoupe, 2007; Oliva-Teles & Gonçalves, 2001). Despite relatively high protein content, *S. cerevisiae* is not used currently as a protein source in commercial fish diets and the literature on high inclusion levels of yeast in diets for fish is rather scarce. *Saccharomyces cerevisiae* contains between 40 and 65% protein with a similar amino acid profile to that of fish meal (Nasseri *et al.*, 2011). However, it can be deficient in some sulphur-containing amino acids such as methionine to a level below the nutrient requirements of most farmed fish (Kuhad *et al.*, 1997).

The yeast *Wickerhamomyces anomalus* is often used in grain bio-preservation and as an anti-mould species due to its ability to outcompete moulds and other yeasts (Tayel *et al.*, 2013; Olstorpe & Passoth, 2011; Fredlund *et al.*, 2004). In addition, *W. anomalus* is characterised by an ability to utilise various substrates for growth and by high phytase activity (Olstorpe *et al.*, 2009; Vohra & Satyanarayana, 2001), making it a potential candidate for improving phosphorus absorption in fish. Its reasonably high protein levels also make it a good candidate for use in animal feeds (Satyanarayana *et al.*, 2012).

The filamentous fungus *Rhizopus oryzae* is commonly grown on spent sulphite liquor, which is a waste product from paper pulp production (Ferreira *et al.*, 2012; Edebo, 2008). It possesses a similar amino acid profile to fish meal (Edebo, 2008; Mydland *et al.*, 2007) and could be a good candidate for replacing fish meal in the diet of fish (Ferreira *et al.*, 2012; Edebo, 2008). *Rhizopus oryzae* also contains polysaccharides, chitosan and chitin (glucosamine and N-acetylglucosamine polymers), integral parts of its cell wall, in variable amounts (Abro *et al.*, 2014). These compounds, when fed to fish can have positive immunomodulatory effects (Harikrishnan *et al.*, 2012; Esteban *et al.*, 2001).

1.5.2 Blue mussel as a protein source

Blue mussel (Mytilus *edulis*) is a filter-feeding marine and brackish bivalve mollusc. It feeds on phytoplankton and thus has the ability to remove excess nitrogen and phosphorus from water. Blue mussel thereby has potential for reducing nutrient loads in eutrophic waters, such as the Baltic Sea (Stadmark & Conley, 2011). Due to its small size, blue mussel from the Baltic Sea is usually not considered attractive for human food market, but could have potential for use in fish and poultry feed. Moreover, blue mussel tends to absorb pollutants from the water, which might render it unsuitable for human or animal diets. However, Lindahl *et al.* (2005) have pointed out that the existence of new techniques and management strategies might eliminate this drawback. Blue mussel is a high quality protein source with high protein levels (Lindahl *et al.*, 2005). The protein possesses a similar amino acid profile to fish meal (Langeland *et al.*, 2014; Berge & Austreng, 1989) and it can be used as a taste enhancer in fish feed (Nagel *et al.*, 2014). However its potential as a substitute for fish meal in diets for fish has not been studied extensively and very few publications exist on this topic.

1.6 Evaluation of novel feed sources

Evaluation of novel feed ingredients is a fundamental step in successful development of novel feed formulations. Nutrient utilisation is defined as the capacity of an animal to utilize the digested nutrients for growth (Glencross *et al.*, 2007). The gastrointestinal tract is the first organ interacting with the feed that acts as an absorptive surface and protective membrane against harmful components. Therefore it is important to assess the digestibility performance for novel feed sources, their uptake and transport rate and the potential effects on intestinal integrity.

1.6.1 Growth and digestibility assessment

Growth measurement is a measure of net nutrient deposition. It is the most common response measured that reflects changes in dietary content of a nutrient. Growth of an animal is influenced by its life stage, genotype, environment and nutrient intake (NRC, 2011). Conventionally, growth measurement in fish nutrition research is assessed by measuring the length and weight of the fish, at the beginning and the end of the experimental period. Moreover, several measuring points may be used during the experiment. Assuming that all fish have started the experiment with the same weight, then the response to different dietary treatments will be reflected in weight gain. Weight gain, or live weight gain (Glencross *et al.*, 2007) can be expressed with

a number of different calculations suitable for different experimental or practical conditions. The most commonly used are weight gain (WG), thermal growth coefficient (TGC), specific growth rate (SGR) and daily growth coefficient (DGC) although other means of expressing growth exist.

Digestibility of a nutrient or energy is a measure that expresses the amount of that nutrient digested by the animal and not excreted from the body. Contemporary diets are mainly formulated based on digestible nutrients and energy rather than gross values (Cho & Kaushik, 1990). Digestibility is of vital importance in developing new feed sources for aquaculture (Glencross *et al.*, 2007). Digestibility can be assessed by the use of direct and indirect methods, with the first relying on total collection of faeces and the latter on the use of inert marker. Estimation of digestibility relies on collecting faeces from an animal and while the total collection of faeces is relatively easy in terrestrial animals, fish are more challenging in this respect. Hence, the indirect method is often used when estimating digestibility in fish. It relies on representative samples from both feed and faeces and the use of inert, indigestible marker. The marker is included in the feed, usually in a small percentage, and is then measured in a representative faeces sample. By calculating the ratio between marker concentration in the feed and faeces, digestibility of dry matter is determined. This is then used for calculating 'apparent digestibility' of other nutrients and energy. The term 'apparent' indicates that this measurement does not discriminate between ingested and endogenously produced nutrients.

1.6.2 Feed utilisation and nutrient retention

Measuring feed utilisation and feed intake in nutrition studies is equally important and complementary to measuring growth. Feed intake is usually expressed as an amount (g fish $^{-}$1) or a rate (g fish^{-1} day^{-1}) and accurate measuring of feed intake in fish is one of more difficult procedures in aquaculture nutrition research (Glencross *et al.*, 2007). Typical ways of expressing feed utilisation are through feed conversion ratio (FCR), feed efficiency and protein efficiency, to name a few. The FCR is expressed as a relationship between consumed feed under a certain period and the live weight gain for that same period. Although these indicators are of high value in nutrition research, their major downside is that they rely on assessment of dry feed weight and live weight of fish and therefore include errors for both assessments (Glencross *et al.*, 2007).

The efficiency of nutrient utilisation can be estimated by assessing the efficiency by which nutrients and energy are retained in the body (Glencross *et al.*, 2004; Cho & Kaushik, 1990). Muscle formation during growth is a reflection of protein deposition and is usually preferred to lipid deposition

(NRC, 2011) but type of the tissue and efficiency of retention are highly dependent on the life stage of the fish. Smaller animals are usually more efficient in nutrient and energy retention than larger and older members of the same species (Glencross *et al.*, 2007; Lupatsch *et al.*, 2003), mainly due to a switch from net protein to net lipid deposition (Kiessling *et al.*, 1991).
Specific calculations used in this thesis are described in section 3, Materials and Methods.

1.6.3 Amino acid metabolism

Proteins are made of amino acids, which are divided into indispensable (essential) and dispensable (nonessential). There are a total of 20 primary amino acids that most microorganisms and plants are able to synthesise. In contrast, animals must acquire some amino acids from their diet, as they are unable to synthesise them. These amino acids are referred to as indispensable (IAA) (Table 1). Conditionally indispensable amino acids are usually provided in the diet in circumstances where rate of utilisation is greater than the rate of synthesis (Li *et al.*, 2009). Dispensable amino acids can be synthesised in the organism from precursors and their content in the diet is not of vital importance. Thus, dispensable amino acids could be deleted from the diet without causing a detrimental effect upon growth of the animal (NRC, 2011).

Structurally, amino acids are molecules that consist of an α amine and an α carboxyl functional group. Their general formula is $H_2NCHRCOOH$, R being the side chain. Amino acids are interconnected via covalent peptide bonds, connecting an α amine functional group of one amino acid and an α carboxyl group of another. Amino acids differ in the nature of their side chains, which can vary in size and structure from being a single hydrogen atom (glycine) to a large heterocyclic group (tryptophan) (NRC, 2011).

Amino acids are not only vital as building blocks of proteins and growth. In fish and terrestrial animals, amino acids regulate a number of metabolic pathways, crucial for maintenance, growth, reproduction and immune responses. These amino acids are often referred to 'functional' amino acids (Li *et al.*, 2009). Amino acids are important in many biological molecules, forming enzymes, precursors for biosynthesis of biological molecules, metabolic intermediates, hormones, neurotransmitters and many other molecules of high metabolic importance (NRC, 2011).

Table 1. *Indispensable, dispensable and conditionally indispensable amino acids for fish and other aquatic animals. Adapted from Li et al. (2009)*

Indispensable AA	Dispensable AA	Conditionally indispensable AA
Arginine	Alanine	Cysteine
Histidine	Asparagine	Glutamine
Isoleucine	Aspartate	Hydroxiproline
Leucine	Glutamate	Proline
Lysine	Glycine	Taurine
Methionine	Serine	
Phenylalanine	Tyrosine	
Threonine		
Tryptophan		
Valine		

During digestion, proteins are hydrolysed and broken down into free amino acids, dipeptides and tripeptides, under the influence of pepsin in the stomach and gastrointestinal enzymes in the intestinal lumen. Amino acids are then transported over the brush border as free amino acids, di-and tri-peptides into the blood stream. Thereafter, they are delivered to various tissues via blood and used for protein synthesis and as precursors for other substances. Metabolism of amino acids is complex and largely integrated with continuous flux within and between the cells (Kaushik & Seiliez, 2010; Wilson & Cowey, 1985).

In comparison with mammals, fish rely extensively on the use of amino acids as an energy source, where a high proportion of amino acids can be used for providing adenosine triphosphate (ATP) via the tricarboxylic acid cycle in the liver (Ballantyne, 2001; Fauconneau & Arnal, 1985). Flow of amino acids into catabolic and anabolic pathways and the mechanism behind this is still not well understood in fish (Ballantyne, 2001).

Some studies have indicated that the value of dietary protein should be estimated based not only on amino acid content, but also on the rate of release and absorption of amino acids during digestion (Yamada *et al.*, 1981). Assessment of plasma free amino acids after a meal indicates metabolic responses in the dynamics between protein intake, tissue metabolism and protein synthesis (Carter *et al.*, 2007). Different free amino acids can appear in blood plasma at various time following their absorption, which can lead to reduction in their utilisation for anabolic purposes and possible use for

catabolic purposes, i.e. covering energy needs (Espe *et al.*, 1993; Yamada *et al.*, 1981).

1.6.4 Dorsal aorta cannulation

Dorsal aorta (DA) cannulation is a surgical technique used frequently as a tool for physiological observations in teleost fish (Djordjevic *et al.*, 2012). It allows for serial blood sampling from single fish, hence eliminating the need for serial slaughter and using large number of fish for obtaining blood. Standard blood sampling usually requires sedation and handling of fish, which triggers a stress response in fish and could alter the intestinal blood flow (Thorarensen *et al.*, 1993). Use of DA cannulation in research on fish is complementary with the 'three Rs', a widely accepted ethics concept in animal experimentation introduced by Russell and Burch (1959), as it allows for a reduction in number of animals used and to some extent, refinement.

Dorsal aorta cannulation was first described by Smith and Bell (1964) although their original method has since been developed and modified several times, namely by Soivio *et al.* (1975), Kiessling *et al.* (1995) and Djordjevic *et al.* (2012). The technique has been used for measurements of blood volume (Conte *et al.*, 1963), evaluation of haematological parameters (Soivio *et al.*, 1975), endocrinology (Bry & Zohar, 1980), post-prandial changes in amino acid (Ok *et al.*, 2001), evaluation of stress response (Djordjevic *et al.*, 2012; Vidakovic *et al.*, 2011) and immunological parameters in fish (Lo *et al.*, 2003).

A number of studies have focused on postprandial free amino acid uptake in fish in response to varying protein sources (Eliason *et al.*, 2010; Ambardekar *et al.*, 2009; Karlsson *et al.*, 2006; Ok *et al.*, 2001; Espe *et al.*, 1993; Yamada *et al.*, 1981). Such studies are a valuable tool in understanding the absorption rate of different amino acids. However, these studies have mostly followed the effects of a single meal in a force fed fish after a prolonged starvation. Force feeding is known to cause stress response in fish and starvation prior to sampling is likely to affect the metabolic pathways of amino acid uptake, indicating a need for research focusing on voluntary fed fish. Very few studies have used DA cannulated, voluntary fed fish to study postprandial changes and those that have reported have mainly focused on Atlantic Salmon (Djordjevic *et al.*, 2012; Mydland *et al.*, 2009; Sunde *et al.*, 2003).

1.6.5 Intestinal barrier function

The intestinal tract and the gastrointestinal barrier are of particular interest when developing new feed sources. The intestinal tract in fish serves a number of important functions such as food digestion, uptake of nutrients and osmoregulation (Sundell & Rønnestad, 2011; Ringø *et al.*, 2003). In order to

maintain digestion and absorption, the physiology of the intestinal wall must function properly (Jutfelt, 2011). Furthermore, the intestinal wall plays an important role as a defence mechanism in fish. This is usually referred to as the gastrointestinal barrier and comprises an extrinsic, intrinsic and immunological barrier (Sundell & Sundh, 2012).

The intrinsic barrier acts as a primary physical barrier between the intestinal lumen and blood circulation. It is made up of the epithelial cell monolayer and tight junction complexes. The gastrointestinal barrier prevents leakage of ions, small sized molecules, translocation of pathogens and allergens into the mucosa and blood circulation (Jutfelt, 2011). Increased leakage and intestinal inflammation is often an indicator of disturbed barrier and may be detrimental for the health and welfare status of the fish (Segner *et al.*, 2012). Additionally, harmful components in feed ingredients can have negative effects on nutrient uptake and barrier function and can cause local inflammation, leading to increased risk of infection and disease susceptibility (Chikwati, 2013; Knudsen *et al.*, 2008).

2 Aims of the thesis

The overall aim of this thesis was to investigate the potential for using various non-human food grade alternative protein sources in future diets for fish species of relevance for Swedish aquaculture. Fungal and mussel meals were evaluated for their effect on growth, digestibility and post-prandial changes in blood metabolites in a series of trials with Arctic charr (*Salvelinus alpinus*), Eurasian perch (*Perca fluviatilis*) and rainbow trout (*Oncorhynchus mykiss*).

Specific aims of Papers I-IV:

- Assess the protein, amino acid and energy digestibility for selected microbial protein source and mussel meal in diets for Arctic charr and Eurasian perch (Paper I).

- Explore further the potential of previously tested microbial protein sources and mussel meal with respect to growth performance and nutrient utilisation in Arctic charr (Paper II).

- Evaluate the effect of microbial protein sources and mussel meal on intestinal barrier function (Paper II).

- Test the grading effect of selected yeast and a new yeast protein source in diets for rainbow trout by evaluating the growth performance, nutrient utilisation and digestibility (Paper III).

- Explore the postprandial profile of free amino acids in rainbow trout fed dietary yeast sources by use of the dorsal aorta cannulation technique (Paper IV).

- Evaluate possible effect on the acute stress response in fish fed high concentration of whole yeast (Paper IV).

3 Materials and methods

3.1 Experimental design

This thesis is based on the four different studies hereafter described in Papers I-IV. The first study (Paper I) was organised as a randomised block design in which five test diets were evaluated on two different fish species, Arctic charr (*Salvelinus alpinus*) and Eurasian perch (*Perca fluviatilis*), in two separate trials. Each diet was given in four replicates.

The second and third studies (Papers II and III) were organised in a fully randomised design with five and eight different test diets and three replicates, evaluated on Arctic charr and rainbow trout, respectively.

The fourth and final study (Paper IV) was organised as a randomised cross-over experiment with three test diets and five replicates, evaluated on rainbow trout.

An overview of the experimental design for all Papers is given in Table 2.

Table 2. *Overview of the experimental design for Papers I-IV*

Paper	I	II	III	IV
Fish species	Arctic charr Eurasian perch	Arctic charr	Rainbow trout	Rainbow trout
Number of diets	5	5	8	3
Replicates	4 per diet	3 per diet	3 per diet	5 per diet
Number of fish[1]	200 300	750	840	15
Period[1]	14 days 21 days	99 days	70 days	28 days
Test ingredients	*Saccharomyces cerevisiae* intact *Saccharomyces cerevisiae* extract *Rhizopus oryzae* *Mytilus edulis*	*Saccharomyces cerevisiae* intact *Saccharomyces cerevisiae* extract *Rhizopus oryzae* *Mytilus edulis*	*Saccharomyces cerevisiae* intact *Wickerhamomyces anomalus/S. cerevisiae* mix	*Saccharomyces cerevisiae* intact *Wickerhamomyces anomalus/S. cerevisiae* mix
Ingredient replacement rate (%)	30% of diet	40% of fish meal on crude protein basis	20, 40 and 60 % of fish meal on digestible protein basis	60% of fish meal on digestible protein basis
Assessment	Apparent digestibility of nutrients	Growth performance and apparent digestibility	Growth performance and digestibility	Blood parameters, stress parameters, amino acid profile

[1] Upper row in Paper I Arctic char, lower row Eurasian perch

3.2 Fish and facilities

The experiment reported in Paper I was conducted at the Department of Animal Nutrition and Management, Swedish University of Agricultural Sciences, Uppsala, Sweden. The Eurasian perch used in the experiment were produced from first-generation wild eggs at Lake Hjälmaren, Sweden by Östgös commercial fish hatchery (Östgös AB, Söderköping, Sweden). The Arctic charr used in the experiment were a product of the 'Arctic superior' Swedish breeding programme (Nilsson *et al.*, 2010) and were raised at Aquaculture Centre North (Kälarna, Sweden).

The fish were kept separated by species at SLU in Uppsala for several months prior to the experiment. For this purpose, 1000 L flow-through tanks

with constant municipal water supply were used. Water temperature, oxygen supply and pH were monitored regularly.

During the experiment, 10 fish per tank of Arctic charr and 15 fish per tank of perch were randomly allocated and kept in 10 90 L PVC tanks, in two separate trials. Trials with both charr and perch consisted of two different experimental periods and the total number of fish used was 200 for charr and 300 for perch. The tanks were connected into a recirculating system equipped with biological, mechanical and UV filtration. Each tank was fitted with waste feed and faeces collectors (Cho *et al.*, 1982).

The experiments presented in Papers II and III were performed at Aquaculture Centre North (Kälarna, Sweden).

The fish used in Paper II were the 'Arctic superior' strain of Artic charr. A total of 750 fish were randomly allocated to 15 square flow-through fibreglass tanks, each 700 L in volume. In the experiment presented in Paper III, a total of 840 rainbow trout were kept in 24 tanks of the same tank type as described above. The tanks were supplied with water from the nearby Lake Ansjön. The rainbow trout used in Paper III were produced locally at Kälarna Research Station.

The experiment presented in Paper IV was conducted at the Department of Animal Nutrition and Management, Swedish University of Agricultural Sciences, Uppsala, Sweden. Rainbow trout were acquired from commercial producer, Vilstena fiskodling AB, Fjärdhundra, Sweden. During the experiment, 15 dorsal aorta (DA) cannulated rainbow trout were individually and randomly distributed in 15 oval 200 L tanks equipped with a flow-through municipal water supply, with controlled water flow and water temperature. In addition, each tank was equipped with an automatic feeder and automatic feed waste collector (Hølland teknologi, Sandnes, Norway).

All experiments were conducted in compliance with laws and regulations on procedures and experiments on live animals in Sweden, overseen by the Swedish Board of Agriculture and approved by the Ethics Committee for Animal Experiments in Sweden.

3.3 Feed ingredients

A total number of five test ingredient was used in all four studies. In Papers I and II, the experimental diets contained intact baker's yeast (*Saccharomyces cerevisiae*) (Jästbolaget®, Stockholm, Sweden), extracted baker's yeast (*S. cerevisiae*) (Alltech Serbia AD, Senta, Serbia), filamentous fungi (*Rhizopus oryzae*) (Cewatech AB, Gothenburg) and blue mussel (*Mytilus edulis*) (Royal Frysk Muscheln GmbH, Emmelsbüll-Hornsbül, Germany). In Papers III and

IV, the experimental diets contained baker's yeast (*S. cerevisiae*) and a mixture of the yeasts *Wickerhamomyces anomalus* and *S. cerevisiae* (Jästbolaget®, Stockholm, Sweden) in a 70:30 ratio. The chemical composition of test ingredients used in Papers I-IV is given in table 3.

Table 3. *Proximate chemical composition (g kg-1 DM) and energy content (MJ kg-1 DM) of test ingredients used in Papers I-IV; intact baker's yeast (*Saccharomyces cerevisiae*), extracted baker's yeast (*S. cerevisiae*), yeast mix (*Wickerhamomyces anomalus/S. cerevisiae*), filamentous fungi (*Rhizopus oryzae*) and blue mussel (*Mytilus edulis*)*

	Feed ingredient				
	S. cerevisiae				
	Intact	Extracted	Yeast mix	*R. oryzae*	*M. edulis*
Crude protein	466	779	422	505	657
Sum of amino acids	428	498	360	274	472
Crude lipid	10	2	9	84	69
Ash	63	153	69	103	89
Gross energy	19.9	18.1	20.4	21,6	22.8
Indispensable amino acids					
Arginine	22.4	16.8	18.8	11.0	36.7
Histidine	10.4	10.4	7.9	9.8	10.2
Isoleucine	22.8	26.4	20.5	17.8	22.0
Leucine	32.1	36.7	28.4	23.3	33.7
Lysine	34.7	38.8	30.3	27.6	38.5
Methionine[1]	9.7	13.0	4.9	2.3	19.2
Phenylalanine	19.3	21.2	17.2	14.5	18.5
Threonine	22.9	20.6	19.5	8.6	21.8
Valine	28.1	32.8	22.2	21.8	24.9
Sum	202.3	216.8	169.8	136.6	225.5
Dispensable amino acids					
Alanine	24.4	37.8	21.5	20.3	25.7
Aspartic acid	45.4	53.4	37.6	25.2	49.7
Cysteine[2, 3]	9.8	12.0	4.0	0.8	14.2
Glutamic acid	66.7	76.3	58.0	35.3	63.3
Glycine	22.1	26.2	18.0	19.7	31.1
Ornithine	1.0	0.0	0.6	8.6	0.3
Proline	15.4	33.8	15.0	3.8	18.5
Serine	18.3	21.1	21.5	15.9	21.3
Tyrosine[3]	22.9	19.6	14.4	8.2	21.8
Sum	225.9	281.3	190.6	137.8	246.0

[1]*Amount present after oxidation of methionine to methionine sulphone.*
[2]*Amount present after oxidation of cysteine and cystine to cysteic acid.*
[3]*Conditionally indispensable (NRC, 2011).*

3.4 Experimental diets

In Paper I, a total of five diets were used; a reference diet with fish meal as the major protein source and four experimental diets consisting of 70% reference diet mash and 30% test ingredient (as-is basis). Experimental diets were formulated as recommended for digestibility studies, according to Cho and Slinger (1979) and contained intact baker's yeast (*S. cerevisiae*), extracted baker's yeast (*S. cerevisiae*), filamentous fungi (*R. oryzae*) and blue mussel (*M. edulis*).

In Paper II, one reference diet and four experimental diets were formulated as iso-nitrogenous and iso-energetic. The reference diet was formulated based on a commercial recipe for Arctic charr with fish meal as the main protein source. The experimental diets were based on a reference diet with 40% of the fish meal replaced on a dry matter (DM) and crude protein (CP) basis with test ingredients. Recommendations on the nutrient requirements of Arctic charr from Jobling *et al.* (1993) were complied with.

In Paper III, a total of one reference and seven experimental diets with graded level of test ingredients were used. Three of the experimental diets contained graded levels of intact baker's yeast (*S. cerevisiae*) replacing 20, 40 and 60% of fish meal on a digestible protein basis and were supplemented with crystalline methionine. Additional diet containing intact baker's yeast (*S. cerevisiae*), replacing 60% of fish meal but without methionine supplementation, was used as a negative control diet. The remaining three experimental diets contained yeast mix (*W. anomalus/S. cerevisiae*) replacing 20, 40 and 60% of fish meal on digestible protein basis. All diets were formulated as iso-nitrogenous and dietary recommendations for rainbow trout from NRC (2011) were followed.

In Paper IV, the experimental diets consisted of the same reference diet and two highest replacement experimental diets as in Paper III.

All experimental diets in Papers I-IV were produced by extrusion at Natural Resources Institute Finland (Laukaa Research Station).

3.5 Dorsal aorta cannulation

Dorsal aorta cannulation in Paper IV was performed according to Soivio *et al.* (1975), as modified by Kiessling *et al.* (1995) and Djordjevic *et al.* (2012).

In brief, all fish subjected to the procedure were previously sedated with 1 mg L^{-1} metomidate (Aquacalm, Western Chemical Inc., Ferndale, USA) until the cessation of avoidance and then anaesthetised in an aerated bath with 80 mg L^{-1} tricaine methane sulphonate (MS222; Finquel, Scan Aqua AS, Årnes,

Norway). Absence of coughing reflex was used for confirmation of anaesthesia. Each fish was then moved in turn onto a V-shaped surgical table and the gills were kept aerated and the fish anaesthetised with a constant recirculating water supply containing 60 mg L^{-1} MS222.
Lidocaine (20 mg mL^{-1}; Xylocain®, AstraZeneca, Södertälje, Sweden) with and without adrenaline (5 µg mL^{-1}; Haukeland Sykehusapotek, Bergen, Norway) was injected into the proximal and distal roof of the mouth respectively, at the planned incision spots. A sterile needle was used for puncturing a hole through the upper jaw through which a 40 mm long polyethylene (PE) 180 cannula with a flat end to act as a stopper was inserted. An incision was made between the first and second gill arch and a 1000 mm long pre-heparinised PE 90 cannula was inserted with the help of guiding wire into the dorsal aorta (DA). The wire was then removed and the PE 90 cannula was guided through a small piece of PE 180 cannula previously placed into the upper jaw. The PE 90 cannula was then guided through the 800 mm long PE 180 sliding cannula sleeve, filled with heparinised saline solution and sealed with heat. The outer PE 180 cannula was used as protection from fish bites. The fish was then weighed, placed in the experimental tank with fresh aerated water and guided in a circular motion against the water current for faster recovery from anaesthesia.

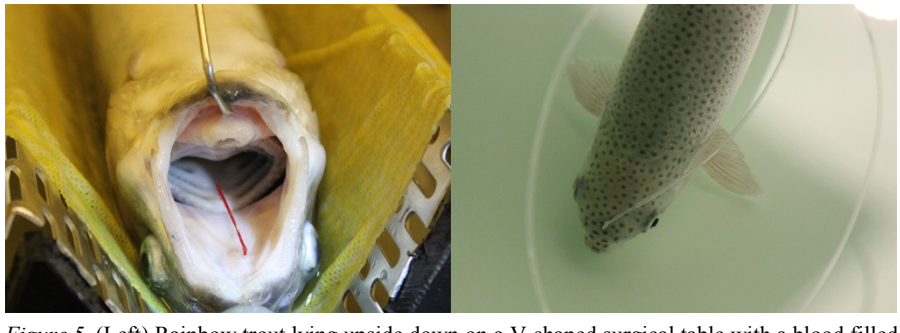

Figure 5. (Left) Rainbow trout lying upside down on a V-shaped surgical table with a blood filled PE 90 cannula. (Right) Rainbow trout swimming in the experimental tank post-surgery, PE-90 cannula with a PE-180 sleeve cannula visible.

3.6 Sample collection

In Paper I, fish faeces were collected using the 'Guelph' faeces collection system in which a 50-mL tube was mounted at the bottom of the vertical settling column attached to each experimental tank. The tanks were drained of any uneaten feed just after feeding and this feed was collected and stored at -25 °C. Faeces were then collected in the 50-mL tube by settling until the next

feeding, when they were collected and centrifuged at 5000g for 10 minutes and the faeces pellet was stored at -25 °C. Each 50-mL tube was immersed in ice in order to minimise microbial activity and dissolution of nutrients in the water.

In Paper II all fish were anesthetized with MS-222 solution (100 mg L^{-1}) at the end of the trial and body weight and length were measured. Five fish per tank were randomly selected and euthanised by an overdose of MS-222 (200 mg L^{-1}) and cutting of the branchial gill arches. Faeces collection in Paper II was performed on these five fish by surgical stripping at the end of the trial. Each fish was eviscerated and the viscera removed for calculation of relative body indices while the faeces were scraped from the distal intestine and pooled for each tank. Five additional fish from each tank were euthanized using the same procedure and stored at -25 °C for whole-body analysis.

In Paper III, faeces were sampled from five fish in week 4, 15 fish in week 7 and 15 fish at the end of the trial. All fish were netted and euthanised at the end of the trial with an overdose of MS 222 and branchial exsanguination as described above. Five fish were stored for later whole-body analysis. All remaining fish from each tank were then sampled for faeces and viscera as described in Paper II. Body weight and length were recorded for each fish. Feed waste in Papers III and IV was collected by belt collectors (Hølland teknologi, Sandnes, Norway) from each tank, weighed and stored at -25 °C for analysis of DM.

In Paper IV, blood samples were taken from each fish at 0, 3, 6, 12 and 24 hours post feeding. The first sample (time 0) was taken just before feeding and sampling was performed on day 7 of every week the fish were fed the experimental diets. Each floating DA cannula was retrieved through a pre-drilled hole in the side of the tank by use of a thin hook (Figure 6). The sealed end of the cannula was then removed and saline solution together with 0.1 mL of blood was removed from the cannula. A blood sample (0.35 mL) was then drawn with the new syringe and the cannula was filled with heparinised saline solution and heat-sealed.

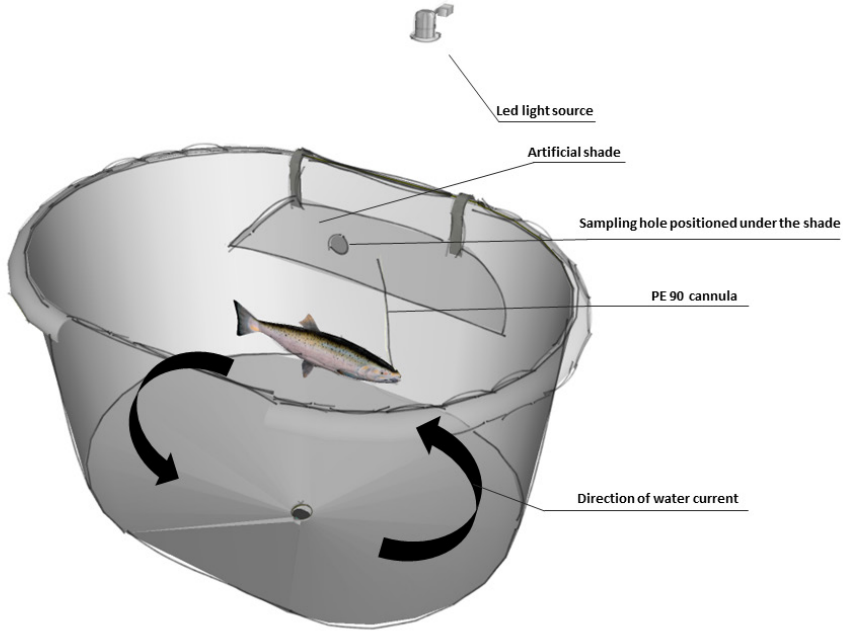

Figure 6. Illustration of the dorsal aorta cannulation tank setup used in Paper IV

3.7 Determination of intestinal barrier function

The Ussing chamber was used in Paper II for assessment of intestinal barrier function on four fish per tank, according to the procedure described by Sundell *et al.* (2003) and modified by Sundell and Sundh (2012). Each fish was anesthetised as described above and euthanised by a blow to the head. Details of the intestinal sampling procedure are provided in Abro *et al.* (2014). In brief, the fish were eviscerated and the intestine from the last pyloric caecum to the anus was removed and opened longitudinally. The intestine was then divided at the ileorectal valve into a proximal and distal part. Blunt dissection was used for removal of the serosa and part of the muscular layers in order to maximise oxygen availability to the intestinal epithelium. These segments were then mounted in Ussing chamber for analysis of intestinal barrier function. More detailed method description is provided in Paper II.

3.8 Chemical analysis

Whole, non-processed fish were stored at -25 °C after sampling and then thawed and homogenised with a mixer (B-400, Büchi Labortechnik AG, Flawil, Switzerland). Homogenised fish, feed and faeces were then freeze-dried, ground with a coffee grinder (KG40, DeLonghi Appliances, Italy) and stored at -25 °C until analysis. Dry matter content (DM) was determined after heating the samples for 16 hours in a ventilated oven at 103 °C. Ash content was determined after incineration at 550 °C for 3 hours. Gross energy content (GE) was analysed in an isoperibol calorimeter (Parr 6300, Parr Instrument Company, Moline, IL, USA). Crude protein content (CP) was determined using the total nitrogen (N) Kjeldahl method and CP was calculated as N x 6.25 (Nordic Committee on Food Analysis, 1976).

Crude lipid content (CL) in Papers I and II was determined using hydrolysation and an extraction unit (Soxtec System HT 1043 Extraction Unit, FOSS Analytical A/S, Hilleröd, Denmark) according to the Official Journal of the European Communities (1984). In Paper III CL content was determined by a similar method using the same equipment but without acid hydrolysis, according to Hooft *et al.* (2011). Titanium dioxide in Papers I-III was analysed according to Short *et al.* (1996).Neutral detergent fibre (NDF) was determined according to Mertens (2002).

Amino acid (AA) determination in Papers I-II was performed using the AccQ·Tag^{TM} method (Waters Corpo-ration, Milford, MA, USA). In brief, samples were hydrolysed with 6M HCl with 1% phenol in a microwave oven (Synthos 3000, Anton Paar Nordic, AB Sweden). For analysis of methionine and cysteine, 50 mg of feed samples were added to 2 mL formic acid:perhydrol (9:1) and incubated overnight at 4 °C. Then, 2 mL 0.17 g mL^{-1} sodium bisulphite solution were mixed into each sample for 15 minutes and the samples were hydrolysed, neutralised, diluted and derivatised. This procedure was performed according to the Waters UPLC® protocol.

Amino acid determination for Paper III and amino acid determination in feed, feed waste and faeces samples for Paper IV were performed at a certified laboratory (Eurofins Food & Agro Testing Sweden AB, Linköping, Sweden) by ion exchange chromatography according to the method by Llames and Fontaine (1994). In brief, after sample oxidation for 16 hours with performic acid, samples were hydrolysed for 23 hours with 6M HCl. Amino acid were separated on an ion-exchange chromatograph (Biochrom 30 amino acid analyser, Biochrom Ltd., Cambridge, England). Identification, integration and quantification of the individual peaks were performed with EZChrom Elite (Biochrom Ltd., Cambridge, England).

Blood plasma amino acids in Paper IV were analysed using the AccQ·Tag^{TM} method with modifications described by Reverter *et al.* (1997). Peaks were identified, integrated and quantified using Empower 2 software (Waters Corporation, Milford, MA, USA). Thereafter, peaks were corrected for dilution to obtain the concentration of amino acid in blood plasma (nmol mL^{-1}). Analysis of whole blood in Paper IV for a number of parameters (Na, K, glucose, pH, PCO_2, TCO_2, HCO_3, base excess and haemoglobin (Hb) was performed using an i-STAT portable clinical analyser (i-STAT Corporation, East Windsor, NJ, USA). Haematocrit (Hct) and leucocrit (Leu) were analysed using the linear and ocular measurement tool after centrifugation of blood at 12 000g for 5 minutes. Red blood cells (RBC) were analysed with a Bürker haemocytometer (Assistent, Glaswarenfabrik Karl Hecht GmbH & Co KG, Sondheim, Germany). Calculation of erythrocyte indices was based on mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC).

3.9 Calculations

Growth performance in Papers II-III was estimated by the use of several indicators, namely weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR) and nutrient retention. These were calculated according to the following equations:

$$\text{WG (\%)} = ((\text{FW} - \text{SW})/\text{SW}) \times 100$$
$$\text{SGR (\% day}^{-1}) = 100 \times ((\textit{ln}\ \text{FW} - \textit{ln}\ \text{SW})/\text{T})$$
$$\text{FCR} = \text{FI}/\text{WG}$$
$$\text{Nutrient retention} = (\text{Nutrient retained in the body}/\text{Nutrient ingested}) \times 100$$

where FW is the final weight (g) of the fish, SW is the initial weight of the fish (g), T is duration of the experiment (days) and FI is total feed intake (g).

Relative body indices (hepatosomatic index (HIS) and viscerosomatic index (VSI)) in Papers II and III were calculated as follows:

$$\text{HSI (\%)} = (W_{Liv}/\text{FW}) \times 100$$
$$\text{VSI (\%)} = W_{Vis}/\text{FW}) \times 100$$

where W_{Liv} is the weight of liver (g), W_{Vis} is the weight of viscera (g) and FW is fish weight.

Apparent digestibility coefficient (ADC) of nutrients and energy in Papers I-III was calculated according to the equation developed by Cho *et al.* (1982):

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

where F is % nutrient (or kJ g^{-1} gross energy) of faeces, D is % nutrient (or kJ g^{-1} gross energy) of diet, D_i is % digestion indicator of diet and F_i is % digestion indicator of faeces.

In Paper I, the ADC of test ingredients was calculated by using the equation developed by Bureau *et al.* (1999):

$$ADC_{test\ ingr.} = ADC_{test\ diet} + [(ADC_{test\ diet} - ADC_{ref.\ diet}) \times (0.7 \times D_{ref.}/0.3 \times D_{test\ ingr.})]$$

where D_{ref} is % nutrient (or kJ g^{-1} gross energy) of reference diet (as-is) and $D_{test\ ingr.}$ is % nutrient (or kJ g^{-1} gross energy) of test ingredient (as-is).

In Papers III and IV, feed intake was calculated as:

Feed given DM (g) – Feed waste DM (g)/Recovery

Feed recovery was calculated as percentage of DM recovered, according to Helland *et al.* (1996).

In Paper IV, a number of blood parameters were used for calculations of different indices. Erythrocyte indices were calculated according to Stoskopf (1993):

MCV = Hct/RBC x 10
MCH = Hb/RBC x 10
MCHC = Hb/Hct x 100

where MCV is mean corpuscular volume, Hct is haematocrit, RBC is red blood cells, MCH is mean corpuscular haemoglobin and Hb is haemoglobin,

3.10 Statistical analysis

In Papers I-III, statistical analysis was performed using Statistical Analysis System version 9.3 (SAS Institute Inc., NC, USA) and, in the case of the Ussing chamber experiment, using IBM SPSS Statistics software version 20 (IBM SPSS Statistics for Windows, Version 20.0.; IBM Corp., Armonk, NY, USA). The significance level was set to $P<0.05$. Data in Paper IV were analysed using statistical software R® version 3.2.2 (R Core Development Team, 2011).

The effect of the experimental diets on ADC in Paper I was evaluated using the model PROC MIXED, followed by Tukey's multiple comparison test. Tank was the experimental unit and the model included the fixed factor of diet and random factor of period.

In Paper II, the same model was used to evaluate the effect of test diets on growth performance and relative organ weights but with a fixed factor of diet and random factor of tank within diet. Furthermore, data analysis for body weight (WG, SW, FW and SGR) was performed without outliers, which were determined as outside the 97.5% confidence interval, in a frequency distribution analysis. Tukey's multiple comparison test was used to adjust for multiple comparisons. Statistical model PROC GLM with a fixed factor of diet was used for the purpose of analysing the effect of diets on FCR, ADC, nutrient and energy retention. Data from the Ussing chamber experiment in Paper II were analysed with the mixed linear model (MLM), with the diet and tank nested within the diet as a fixed factor. Sidak-adjusted pair-wise comparisons of the estimated marginal means of experimental diets to control diets were used for subsequent *post hoc* analysis.

In Paper III, the effect of test diet on growth performance, nutrient retention and relative organ weight was analysed with PROC MIXED model. The fixed factor of diet and daily feed intake (DFI) and the random factor of tank within test diet were used. The effect of experimental diet on ADC and DFI was assessed with PROC MIXED model, with the fixed factor of test diet and the random factor of tank within test diet. Tank was the experimental unit and Tukey's multiple comparison test was used.

In Paper IV, using statistical software R® (R Core Development Team, 2011), data were analysed with Linear Mixed Effects (lme) model. Fixed effects included in the models were diet, hour, feed intake (% BW/day) and fish weight. Random effects in the model were fish and week. Interaction between diet and hour was included in the models. In addition, correlation between hour and fish-week was used in the lme models to account for repeated measures on the same fish. Least square mean (lsmeans) tests were used for determination of significant differences between the effect of diet and

stress. Tukey's pairwise comparison test was used. Normality was tested with normal probability plot (qqnorm) and all results were considered significant at $P<0.05$.

4 Summary of main results

4.1 Chemical composition of test ingredients and feed(Papers I-IV)

Test ingredients used in all four studies and their chemical composition are shown in Table 3.

The crude protein content of test ingredients used in Papers I-IV varied from 422 to 779 g kg^{-1} DM and was highest for extracted *S. cerevisiae*, followed by *M. edulis*, *R. oryzae*, intact *S. cerevisiae* and the *W. anomalus*/*S. cerevisiae* mix. The filamentous fungi, *R. oryzae*, which was used in Papers I and II, was produced in two different batches and the chemical composition varied slightly due to production conditions. The first batch, which was used in Paper I, had lower crude protein content (479 g kg^{-1} DM) than the second batch which was used in Paper II (505 g kg^{-1} DM The ingredients did not vary greatly with respect to total amino acid content, with the exception of *R. oryzae*, which had the lowest amount of amino acids. Moreover, the highest indispensable amino acid content was detected in *M. edulis* and the lowest in *R. oryzae*. In general, the content of sulphur-containing amino acids, especially methionine, was highest in *M. edulis* (19.2 g kg^{-1} DM) and lower in ingredients of microbial origin. Crude lipid content varied considerably, with the highest content in *R. oryzae* and *M. edulis* and the lowest in extracted *S. cerevisiae.* Similarly, neutral detergent fibre content was highest in *R. oryzae* and lowest in intact and extracted *S. cerevisiae.* Ash content varied between 63 and 153 g kg^{-1} DM, with the highest value recorded in extracted *S. cerevisiae* and the lowest in intact *S. cerevisiae.* Gross energy content varied between 18.1 and 22.8 MJ kg^{-1} DM with the highest content in *M. edulis* and the lowest in extracted *S. cerevisiae.*

The crude protein content of the experimental diets in Paper I varied from 483 g kg^{-1} DM (diet with *R. oryzae*) to 600 g kg^{-1} DM (diet with extracted *S. cerevisiae*) (Table 4). Total sum of amino acids varied from 344 to 470 g kg^{-1} DM, with the lowest level in the diet with *R. oryzae* and the highest in the diet with *M. edulis*. Highest crude lipid content was recorded for the reference diet and lowest for the diet with extracted *S. cerevisiae*. Neutral detergent fibre content was highest in the diet with *R. oryzae* and lowest in the diet with intact *S. cerevisiae*. Gross energy content varied from 21.4 MJ kg^{-1} DM (diet with extracted *S. cerevisiae*) to 23.8 MJ kg^{-1} DM (reference diet).

Indispensable amino acid content varied between diets, with the concentrations of the limiting amino acids lysine and methionine being in highest in the diet with *M. edulis* and lowest in the diet with *R. oryzae* (Table 4).

In Paper II, the diets varied only slightly in respect to chemical composition and energy content (Table 4). Crude protein content varied between 480 (diet with *R. oryzae*) and 498 g kg^{-1} DM (diet with *M. edulis*) while the total sum of amino acids ranged between 439 (reference diet) and 500 g kg^{-1} DM (diet with extracted *S. cerevisiae*). Gross energy content was highest for the diet with *M. edulis* and lowest for the diet with extracted *S. cerevisiae*.

Similarly to Paper II, the diets used in Papers III and IV were formulated to be iso-nitrogenous and iso-energetic and were reasonably similar in terms of chemical composition (Table 5). Crude protein content was lowest in the reference diet (425 g kg^{-1} DM) and highest in the diet with high inclusion of yeast mix (463 g kg^{-1} DM). Sum of amino acids varied from 366 g kg^{-1} DM (diet with low inclusion of yeast mix) to 416 g kg^{-1} DM (diet with high *S. cerevisiae* content and no methionine). Crude lipid content was between 186 and 208 g kg^{-1} DM while gross energy content ranged from 23.6 to 23.9 MJ kg^{-1} DM. The neutral detergent fibre content was lowest in the diet with the high inclusion of yeast mix (25.4 g kg^{-1} DM) and highest in the reference diet (113.9 g kg^{-1} DM). Phosphorus content was highest in the diet with low inclusion of yeast mix and lowest in the reference diet.

Table 4. Proximate chemical composition (g kg^{-1} DM), energy content (MJ kg^{-1} DM) and amino acid content (g kg^{-1} DM) of the experimental diets in Papers I and II

	Experimental diet Paper I[1]					Experimental diet Paper II[1]				
	REF	MYE	ISC	ESC	RHO	REF	MYE	ISC	ESC	RHO
Dry matter (%)	95.3	95.4	92.1	91.7	92.7	91.2	91.7	91.3	92.9	90.8
Crude protein	490	540	485	600	483	493	498	492	494	480
Sum of amino acids	417	470	369	436	344	439	465	491	500	443
Crude lipid	230	187	167	113	194	201	201	190	174	186
Neutral detergent fibrer	61	46	37	43	75	-	-	-	-	-
Ash	84	84	74	110	84	76	74	67	75	73
Gross energy	23.8	23.2	22.7	21.4	22.8	24.1	24.4	23.9	23.2	23.9
Indispensable amino acids										
Arginine	24.6	30.5	21.1	21.9	19.8	28.1	30.6	28.4	27.5	25.3
Histidine	8.8	10.5	8.9	10.3	8.5	11.0	10.4	12.1	12.0	12.6
Isoleucine	19.5	22.4	17.3	21.3	16.4	21.4	19.5	23.4	23.4	22.8
Leucine	33.9	37.2	29.1	33.7	27.3	36.4	35.7	38.6	38.2	35.5
Lysine	28.1	34.2	25.9	29.7	23.2	31.6	33.0	34.0	34.3	32.5
Methionine[2]	15.9	18.2	13.6	17.2	13.6	18.4	14.2	13.4	15.7	14.1
Phenylalanine	18.8	20.5	16.6	19.5	15.5	20.1	20.3	22.5	22.0	20.7
Threonine	16.7	19.8	15.8	17.4	14.6	19.5	20.7	20.7	19.4	15.4
Valine	23.8	26.5	21.4	26.1	20.1	26.3	23.9	28.6	28.4	27.5
Sum	190.2	219.9	169.8	197.1	159.0	212.7	208.4	221.6	220.8	206.3

[1]REF = reference diet, MYE = diet with blue mussel (*Mytilus edulis*), ISC = diet with intact yeast (*Saccharomyces cerevisiae*), ESC = diet with extracted yeast (*S. cerevisiae*), RHO = diet with filamentous fungi *Rhizopus oryzae.*
[2]Amount present after oxidation of methionine to methionine sulphone.

Table 5. *Proximate chemical composition (g kg^{-1} DM), energy content (MJ kg^{-1} DM) and amino acid content (g kg^{-1} DM)of the experimental diets in Papers III and IV (diets FM, S60 and W60 were used in Paper IV and correspond to FM, SC and WS, respectively)*

	Experimental diet[1]							
	FM	**S20**	**S40**	**S60**	**S60-Met**	**W20**	**W40**	**W60**
Dry matter (%)	92.4	91.1	91.9	91.3	90.4	91.8	92.3	93.3
Crude protein	425	433	440	454	453	432	446	463
Total amino acids	387	389	392	382	416	366	399	393
Crude lipid	196	207	208	203	192	208	200	186
NDF[2]	113.9	88.2	63.9	44.9	44.9	81.7	68.0	25.4
Ash	68.4	66.4	62.8	62.6	59.6	65.2	63.2	61.6
Gross energy	23.6	23.6	23.7	23.9	23.9	23.6	23.6	23.8
Phosphorus	9.3	9.7	9.8	9.8	10.1	10.7	10.1	9.8
Sodium	5.6	4.8	3.8	3.0	2.8	5.5	4.1	2.8
Potassium	8.1	9.6	11.0	12.7	13.4	11.1	11.6	11.9
Indispensable amino acids								
Arginine	22.1	22.4	22.0	21.3	22.6	20.4	21.9	21.7
Histidine	9.5	9.3	9.5	9.4	9.7	8.8	8.9	9.4
Isoleucine	16.5	16.8	16.8	16.9	18.5	16.1	17.2	17.4
Leucine	30.1	30.3	29.5	28.7	31.9	29.0	30.6	30.2
Lysine	24.1	24.4	24.6	24.4	26.6	22.6	24.4	24.3
Methionine	11.1	12.2	11.4	11.6	7.8	11.3	12.4	12.3
Phenylalanine	18.6	18.9	19.0	18.9	20.8	17.6	19.8	19
Threonine	15.4	15.5	16.3	15.7	16.3	14.3	16.3	16.3
Valine	19.6	19.6	19.9	19.6	21.2	18.2	19.6	20.6
Sum	167.0	169.4	169.0	166.5	175.4	158.3	171.1	171.2

[1]FM=fish meal-based reference diet, S20=diet with 20% of fish meal replaced with *Saccharomyces cerevisiae*, S40=diet with 40% of fish meal replaced with *S. cerevisiae*, S60=diet with 60% of fish meal replaced with *S. cerevisiae*, S60-Met=diet with 60% of fish meal replaced with *S. cerevisiae* and no added crystalline methionine, W20=diet with 20% of fish meal replaced with *Wickerhamomyces anomalus/S. cerevisiae* mix, W40=diet with 40% of fish meal replaced with *W. anomalus/S. cerevisiae* mix, W60=diet with 60% of fish meal replaced with *W. anomalus/S. cerevisiae* mix

[2]NDF = Neutral detergent fibre

4.2 Apparent digestibility (Papers I-III)

For Arctic charr in Paper I, diets with extracted *S. cerevisiae*, *R. oryzae* and *M. edulis* had higher apparent digestibility than the reference fish meal based diet and the diet with intact *S. cerevisiae*. Digestibility of crude protein was higher for the diet with extracted *S. cerevisiae* than for the diet with intact *S. cerevisiae*, while there were no differences between the other diets. Diets with *M. edulis* and extracted *S. cerevisiae* generally had the highest digestibility of sum of amino acids and most indispensable amino acids. Apparent digestibility of gross energy was higher for diets with extracted *S. cerevisiae*, *R. oryzae* and *M. edulis* than for intact *S. cerevisiae.*

Furthermore, analysis of apparent digestibility of test ingredients in diets for Arctic charr revealed consistently lower digestibility of intact *S. cerevisiae* than of other test ingredients. This was found for dry matter, sum of amino acids, gross energy and most indispensable amino acids, but not for crude protein. On the other hand, digestibility values of extracted *S. cerevisiae*, *R. oryzae* and *M. edulis* were rather high, reaching values close to 100% for most nutrients.

For Eurasian perch in Paper I, the diet with extracted *S. cerevisiae* had higher apparent digestibility of isoleucine, leucine, threonine and valine than the diet with *R. oryzae*. There were no effects of dietary treatment on apparent digestibility of dry matter, crude protein or sum of amino acids. Moreover, there were no effects of dietary treatment on ingredient digestibility in Eurasian perch.

The apparent digestibility of dry matter in diets for Arctic charr (Paper II) was higher for the diet with *M. edulis* than for the reference diet and the diets with intact *S. cerevisiae* and *R. oryzae.* The apparent digestibility of crude protein was highest for the reference diet, followed by *M. edulis* and extracted *S. cerevisiae* diets. There were no effects of dietary treatment on gross energy digestibility. The highest apparent digestibility for total and indispensable amino acids was found for the diets with extracted *S. cerevisiae*, *M. edulis* and the reference diet. The diet with intact *S. cerevisiae* had consistently lower digestibility for most amino acids than the diet with extracted *S. cerevisiae*. In addition, the diet with intact *S. cerevisiae* had lower digestibility of lysine, methionine and threonine than the reference diet and the diet with *M. edulis.*

In Paper III, there was no effect of dietary treatment on dry matter digestibility. Apparent digestibility of crude protein and sum of indispensable amino acids was highest for the reference diet and the diet with 20% yeast mix. For most of the individual indispensable amino acids, digestibility was highest

for the reference diet and the diet with 20% yeast mix with the exception of threonine, for which digestibility was highest in the reference diet. Apparent digestibility of phosphorus was highest for the diet with 20% yeast mix and lowest for the reference diet.

4.3 Growth performance and nutrient utilisation (Papers II-III)

In Paper I, Arctic charr achieved a weight gain ranging from 3.6% (diets with *R. oryzae* and extruded *S. cerevisiae*) to 17.2% (diet with intact *S. cerevisiae*). Weight gain of Eurasian perch varied from 2.4% (diet with *R. oryzae*) to 18.6% (diet with *M. edulis*). Diets used in Paper I were designed in accordance with the protocol described by Cho and Slinger (1979), which involves mixing the reference diet and test ingredient in a 70:30 ratio. Consequently, test diets were not intended for maximising growth performance, but purely for analysing apparent digestibility.

In Paper II, growth performance did not differ between the fish fed the reference diet, the diet with intact *S. cerevisiae* and the diet with *M. edulis* in terms of final weight, specific growth rate and weight gain. However, fish fed the diets with extracted *S. cerevisiae* and *R. oryzae* had significantly lower final weight, specific growth rate and weight gain. Dietary treatment had no significant effect on feed conversion ratio although fish fed the diet with *R. oryzae* had a tendency (P=0.064) for higher feed conversion ratio than fish fed the reference diet. Crude protein retention (N x 6.25) was higher in fish fed the reference diet, than in fish fed the diet with extracted *S. cerevisiae*. However, there were no other differences in crude protein retention between fish fed the reference diet and any of the remaining test diets. For the sum of amino acids, retention was higher in fish fed the diet with extracted *S. cerevisiae* than in fish fed the reference diet and the diet with *M. edulis*. For retention of indispensable amino acids, diet had a significant effect for isoleucine, lysine and valine. However, when using adjustment for pair-wise comparisons, no differences were found between individual dietary treatments for any of the individual amino acids. No effect of dietary treatment was seen on retention of gross energy.

In Paper III, graded levels of the two yeast protein sources in diets for rainbow trout resulted in no difference between fish in different dietary treatments, with respect to final weight. However, there was a tendency (P=0.06) for lower final weight in fish fed diets with the highest inclusion levels of intact *S.cerevisiae* (S 60 and S60-Met). In addition, there was a tendency for lower weight gain in fish fed the diet with highest inclusion of *W. anomalus*/*S. cerevisiae* mix, compared with fish fed the reference diet and

those fed the diets with the lowest inclusion level of *S. cerevisiae* and of *W. anomalus*/*S. cerevisiae* mix. Methionine supplementation did not seem to affect final weight. Specific growth rate was lower for fish fed the diet with the highest inclusion level of *W. anomalus*/*S. cerevisiae* mix for all experimental periods. However, when specific growth rate was studied for different weighing periods during the experiment, there was a clear effect of change in feeders for the last weighing period. This was illustrated by the highest specific growth rate for fish fed diets with the highest inclusion rate of *S. cerevisiae* and *W. anomalus*/*S. cerevisiae* mix.

For Paper III, there were no differences between dietary treatments with respect to feed conversion ratio. Results for relative body index revealed higher hepatosomatic index for fish fed the diet with the lowest inclusion ratio of *W. anomalus*/*S. cerevisiae* mix than for fish fed the diet with medium inclusion of *W. anomalus*/*S. cerevisiae* mix. In terms of viscerosomatic index, there were no differences between dietary treatments.

Retention values for crude protein, crude lipids, total amino acids, gross energy and phosphorus were unaffected by dietary treatment. However, there were clear numerical differences in crude lipid retention between fish fed the reference diet (89.77%) and fish fed the diet with the highest inclusion of *S. cerevisiae* (74.65%).

4.4 Active transport and intestinal barrier function (Paper II)

In Paper II, analyses of active transport in the proximal intestine revealed lower short-circuit current and higher trans-epithelial potential for fish fed the diet with *M. edulis* than for fish fed the reference diet. There were no differences in these parameters for the distal intestine between the dietary treatments.

In the distal intestine, the uptake of lysine was higher for the diet with intact *S. cerevisiae* than for the reference diet.

Apparent permeability as a function of intestinal barrier function was higher for the diets with intact *S. cerevisiae* and *R. oryzae* than for the reference diet. There were no significant differences between diets for trans-epithelial resistance in the proximal intestine. However, there was a tendency ($P= 0.07$) for lower trans-epithelial resistance in the distal intestine of fish fed diets with *M. edulis* and *S. cerevisiae* compared with fish fed reference diet.

4.5 Amino acid profiles in plasma (Paper IV)

In Paper IV, analysis of post-prandial changes in plasma free amino acids revealed a difference between dietary treatments with respect to methionine (indispensable). The concentration of methionine was significantly lower in fish fed the reference diet than in fish fed the two experimental diets for all post-prandial sampling points (0, 3, 6, 12 and 24 hours). Although there were no differences for other indispensable amino acids between the treatments, post-prandial levels of plasma free hydroxy-lysine-2 (dispensable), 3-methyl-histidine (endogen) and hydroxy-proline (endogen) in fish fed the reference diet varied significantly compared with those in fish fed the diets with *S. cerevisiae* and *W. anomalus*/*S. cerevisiae.*

In most cases, plasma free amino acid level peaked at 6 hours and returned to initial levels at 24 hour. Plasma indispensable amino acids peaked significantly at 3, 6 or 12 hours, while there were no significant post-prandial changes in dispensable and endogenous amino acids between sample times.

4.6 Whole blood parameters (Paper IV)

Post-prandial whole blood levels of pH, TCO_2, HCO_3 and base excess increased significantly for fish fed both experimental diets in Paper IV compared with fish fed the reference diet. Mean corpuscular haemoglobin (MCH) level was higher for fish fed the diet with *W. anomalus*/*S. cerevisiae* than for fish fed the reference diet. After the fish were stressed, the levels of pH, TCO_2, HCO_3 and base excess decreased significantly while glucose increased significantly. In general, TCO_2, HCO_3 and base excess peaked at 3 hours, while Hct, Leu and Hb peaked at 0 hours with a steady decline until after 24 hours. Glucose and K peaked at 6 and 0 hours respectively, in fish fed the reference diet, and at 12 and 3 hours respectively, in fish fed the diet with *S. cerevisiae* and the diet with *W. anomalus*/*S. cerevisiae.*

In the stress test, glucose blood levels followed the same profile as in the diet test, whilst the concentration was two-fold higher. Other parameters, such as pH, TCO_2, HCO_3 and base excess followed a different pattern than in the diet test. The lowest concentration was observed at 3 hours while the peak was recorded at 6 hours. Hct, Leu and Hb peaked at 3 hours in stressed fish rather than consistently decreasing as in the diet test.

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5 General discussion

5.1 Chemical composition of test ingredients

Analysis of the ingredients used in Papers I-IV revealed a relatively high protein content, but it was still lower than that of fish meal (745 g kg^{-1} DM) for all test ingredients with the exception of extracted *S. cerevisiae* (779 g kg^{-1} DM). This may pose a limitation to the use of these ingredients in fish diets, as the inclusion level in diets would have to be high in order to meet the protein requirement of the fish.

In terms of indispensable amino acid content, intact *S. cerevisiae* and *W. anomalus/S. cerevisiae* mix were the most similar to fish meal, expressed as percentage of protein (Figure 7). However, methionine content in both extracted and intact *S. cerevisiae*, *W. anomalus/S. cerevisiae* mix and *R. oryzae* was lower than in fish meal, confirming previous findings by Kuhad *et al.* (1997) and Øverland *et al.* (2013). Hence, methionine supplementation should be considered when using these ingredients in diet formulations.

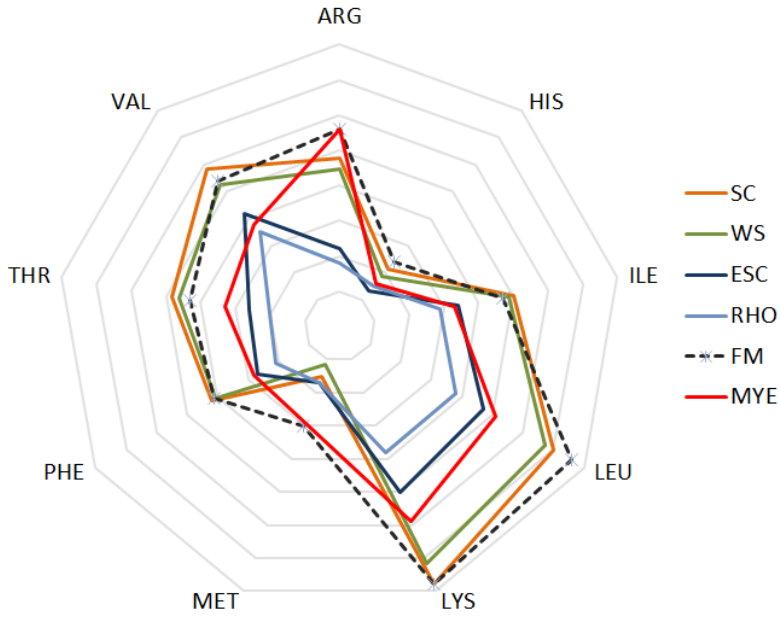

Figure 7. Comparative indispensable amino acid profile of the main protein sources used in Papers I-IV. SC- intact *Saccharomyces cerevisiae*, WS-*Wickerhamomyces anomalus/S. cerevisiae* mix, ESC- extracted *S. cerevisiae*, RHO- *R. oryzae*, FM- fish meal, MYE-*M. edulis*

5.2 Apparent digestibility

In the experiment with Arctic charr presented in Paper I, inclusion of extracted *S. cerevisiae* and blue mussel *M. edulis* in the diets had positive effects on overall diet digestibility. Apparent digestibility values for dry matter (DM), crude protein (CP), gross energy (GE), sum of amino acids and most indispensable amino acids were all positively affected with inclusion of these ingredients. These results were later confirmed in Paper II, when diets with the same ingredients were fed to Arctic charr for 14 weeks.

The higher digestibility of diets with extracted *S. cerevisiae* compared with intact *S. cerevisiae* might be explained by the absence of cell walls in extracted *S. cerevisiae.* During production of the yeast extract, cell walls are removed by autolysis. Presence of yeast cell walls has been shown to have a negative effect on diet digestibility in several fish species (Rumsey *et al.*, 1991; Rumsey *et al.*, 1990). In addition, yeast autolysis may have resulted in an increased amount of short peptides and free amino acids due to hydrolysis. These protein building

blocks are more easily absorbed but may have caused an imbalance in amino acid absorption, possibly leading to their catabolism and use for energy instead of growth. This would explain the high digestibility of the diet with extracted S. *cerevisiae*, but accompanied by low growth and poor retention of nitrogen and amino acids, observed in Paper II. Berge and Austreng (1989) used graded levels of blue mussel *M. edulis* in diets for rainbow trout and showed that at the same inclusion level as used in Paper I, apparent digestibility of DM decreased significantly. However, they used whole blue mussels in the diets and later attributed the decrease in digestibility of DM to the increasing shell fraction. In the work described in this thesis, *M. edulis* was previously de-shelled which may explain the higher digestibility observed in Papers I and II.

The diet supplemented with *R. oryzae* in Paper I showed improved digestibility of DM compared with the reference diet and the diet with intact *S. cerevisiae.* However, in Paper II, the diet with *R. oryzae* had lower digestibility of DM, CP and GE than all other diets. This discrepancy could be a consequence of using two different batches of *R. oryzae* in Papers I and II. Furthermore, the consistency of the faeces collected from Arctic charr fed the diet with *R. oryzae* in Paper II was to some extent liquid. The producer of the *R. oryzae* biomass stated that the quality may fluctuate between batches and that spent sulphite liquor, which is used in production of *R. oryzae,* may contain high quantities of magnesium sulphate ($MgSO_4$). Earlier studies have shown that $MgSO_4$ can induce diarrhoea in rats and mice (Uddin *et al.*, 2005; Izzo *et al.*, 1994) and this could to some extent be have caused the low digestibility of *R. oryzae* in Paper II.

In the experiment with Eurasian perch presented in Paper I, there were no significant differences between different diets in terms of apparent digestibility. Perch demonstrated higher values of digestibility of DM and GE for diets with intact *S. cerevisiae* than the Arctic charr in Paper. Furthermore, the digestibility of certain amino acids in perch seemed to follow an opposing trend of that in charr. One possible explanation for this discrepancy is differences in gastrointestinal enzymatic activity between perch and charr. Langeland *et al.* (2013) showed that digestive enzyme activity in Arctic charr and Eurasian perch differs substantially with respect to number of digestive enzymes.

In Paper III, the highest apparent digestibility values of DM, CP, total and most individual indispensable amino acids were observed for the reference diet and the diet with low inclusion of *W. anomalus/S. cerevisiae* mix, indicating greater potential use of *W. anomalus/S. cerevisiae* mix than intact *S. cerevisiae* in diets for rainbow trout. Although the apparent digestibility of DM, CP and total IAA in Paper III seemed to decrease with increasing inclusion rate of each

test ingredient, this effect was not significantly different between test diets with low, medium and high inclusion. However, there were significant differences between dietary treatments with respect to certain IAA. Methionine digestibility was lowest in the diet with high inclusion of intact *S. cerevisiae* and no methionine supplementation, which is in agreement with results from Paper II. This indicates that diet supplementation with crystalline methionine improved overall methionine digestibility, which is in agreement with findings by Sveier *et al.* (2001).

Table 6. *Apparent digestibility of crude protein and dry matter for different protein sources used in Papers I and II*

	Reference	Intact *S. cerevisiae*	Extracted*S. cerevisiae*	*R. oryzae*	*M. edulis*
Crude protein					
Paper I (charr)	88^{ab}	86^{a}	93^{b}	90^{ab}	92^{ab}
Paper I (perch)	95	94	96	93	95
PaperII	87^{a}	84^{b}	90^{a}	80^{c}	88^{a}
Dry matter					
Paper I (charr)	81^{a}	78^{a}	87^{b}	86^{b}	86^{b}
Paper I (perch)	90	88	92	90	90
Paper II	70^{a}	71^{a}	72^{ac}	62^{b}	74^{c}

Valueswith different superscripts are significantly different ($p<0.05$)

Interesting observation relating to Papers II and III was the somewhat low apparent digestibility in comparison with other IAA for threonine in the diets with intact *S. cerevisiae* and *R. oryzae* in Paper II, and the diets with the highest inclusion of *S. cerevisiae* and of *W. anomalus/S. cerevisiae* mix in Paper III. At the same time, fish fed the diet with intact *S. cerevisiae* in Paper II and those fed the diet with the highest inclusion levels of *S. cerevisiae* and of *W. anomalus/S. cerevisiae* mix in Paper III produced faeces of liquid consistency. Differences in faeces DM content in Paper III are shown in Figure 8. In fish fed diets with *R. oryzae* in Paper II, the faeces were also liquid and the possibility of diarrhoea induced by the presence of $MgSO_4$ in *R. oryzae* has already been discussed.

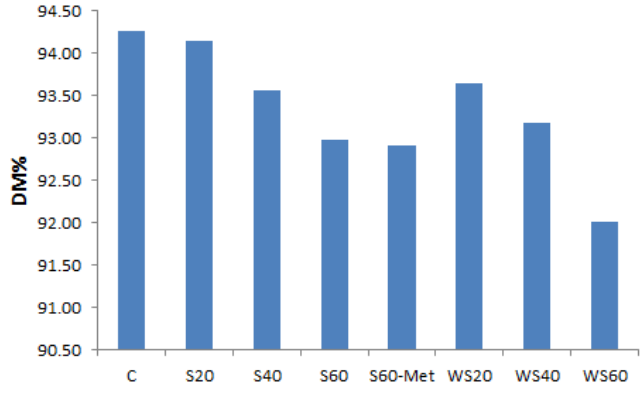

Figure 8. Percentage of dry matter in faeces of rainbow trout in Paper III

Threonine is present in high amounts in mucins in fish (NRC, 2011) and, as a consequence, is excreted in higher amounts by fish during stress-induced mucus over-production (Khan & McGeer, 2013; Eddy & Fraser, 1982). This reduction in apparent digestibility may indicate alterations in the gut mucosa of fish fed these diets, a consequent increase in mucus production and increased levels of threonine in the faeces.

In Paper III, the apparent digestibility of phosphorus varied between the dietary treatments and was highest for the diet with low inclusion of *W. anomalus/S. cerevisiae* mix and lowest for the reference diet. This may indicate an effect of phytase activity by *W. anomalus* yeast.

Apparent digestibility results for the individual test ingredients used in Papers I-III were in agreement in relative terms, but the actual values were generally higher in Paper I. It has been reported previously that the choice of faeces collection method can affect the actual values, e.g. the Guelph collection method which is based on settling of particles, is known to cause overestimation of digestibility, while dissection and stripping can cause slight underestimation (Hajen *et al.*, 1993). In addition, digestibility values for intact *S. cerevisiae* in Papers I-III were higher than those previously reported in several studies (Øverland *et al.*, 2013; Oliva-Teles & Gonçalves, 2001; Rumsey *et al.*, 1991). This may be due to the feed production method, since the diets in Papers I-III were produced by extrusion, as opposed to the cold pelleting used in previous studies.

5.3 Growth performance and nutrient utilisation

The results obtained in Paper II indicate that both *M. edulis* and intact *S. cerevisiae* can be used to replace 40% of fish meal on a crude protein basis without negative effects on growth performance and feed conversion. On the other hand, diets supplemented with extracted *S. cerevisiae* and *R. oryzae* resulted in lower growth performance (Table 7).

The data on retention of IAA in Paper II showed increased methionine retention in diets with intact *S. cerevisiae* and *M. edulis,* possibly due to the lower methionine content in these diets.

These results contradict earlier findings in studies involving the use of intact *S. cerevisiae* in diets for salmonid fish. For example, Øverland *et al.* (2013) found that both apparent digestibility and the growth of Atlantic salmon were negatively affected when 40% of fish meal on a crude protein basis was replaced with intact *S. cerevisiae.* Similarly, Rumsey *et al.* (1990) suggested that cell wall presence in intact *S. cerevisiae* can negatively affect growth and digestibility in lake trout (*Salvelinus namaycush*). However, the ingredients used in the diets tested in Papers I-IV were all produced by extrusion, as opposed to the cold pelleting used by Rumsey *et al.* (1990) and Øverland *et al.* (2013). Positive effects of extrusion on digestibility and growth in various fish species fed different protein sources have been documented previously (Barrows *et al.*, 2007; Venou *et al.*, 2006; Venou *et al.*, 2003; Booth *et al.*, 2002). Hence, extrusion could potentially cause partial disruption of yeast cell walls, thereby increasing the bioavailability of protein and amino acids.

In the case of *M. edulis*, there are few previous studies involving its use as a fish meal substitute in fish diets. Berge and Austreng (1989) used whole, ground *M. edulis* in diets for rainbow trout and concluded that despite clear potential as a protein source, increasing levels of *M. edulis* reduce energy density and increase ash content in the diets, hence driving the apparent digestibility down. However, the results from Paper II clearly show that when de-shelled *M. edulis* is added in the diets to Arctic charr, none of these issues exists.

In Paper III growth performance was unaffected by dietary treatment in terms of final weight and weight gain (Table 8). Test diet formulation in Paper III was performed by replacing fish meal with test ingredients on a digestible protein basis, based on apparent digestibility results for intact *S. cerevisiae* in Paper II. This resulted in higher crude protein content in the test diets than in the reference diet, which may have compensated for differences in growth performance. Murray and Marchant (1986) and Hauptman *et al.* (2014) demonstrated improved growth performance in rainbow trout fed fungal protein sources supplemented with methionine. However, there was no positive

effect of methionine supplementation on growth performance in Paper III. Methionine content in diet S60-Met (7.8 g kg^{-1} DM) was still above the minimum requirement for rainbow trout (7 g kg^{-1} DM), despite no methionine supplementation which might have contributed to the lack of differences in growth performance.

The tendency for lower final weight and lower weight gain observed for the diets with the highest inclusion of both *S. cerevisiae* and *W. anomalus/S. cerevisiae* mix may indicate possible limitations in the use of such high inclusion levels of yeasts in diets for rainbow trout.

Moreover, the results in Papers I and III indicate a potential issue in relation to extrusion of diets containing high inclusion levels of yeast. It was noted that the diets with extracted *S. cerevisiae* in Paper I, and the diets with 60% fish meal replaced with yeasts in Paper III did not properly absorb the oil during vacuum coating. In Paper III, this led to clogging of the drum feeders used, thus decreasing the feed allowance to fish with the highest yeast inclusion levels (Table 8).

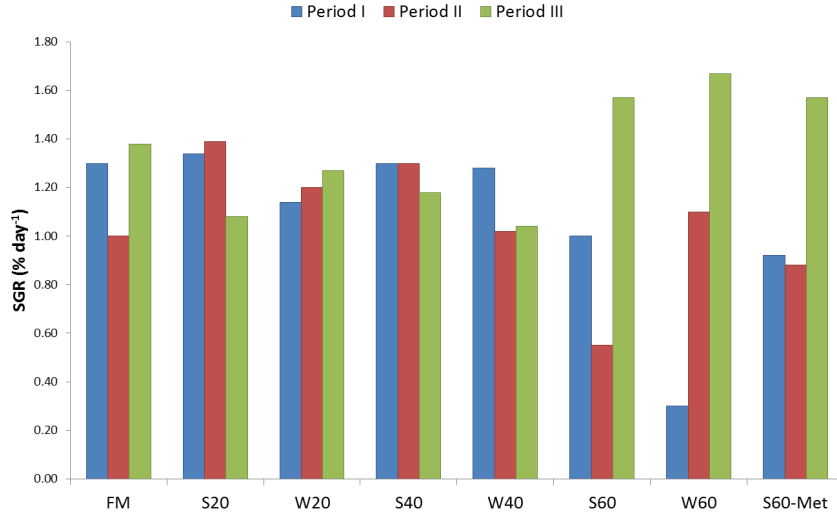

Figure 9. Specific growth rate (SGR) of rainbow trout in Paper III, divided by period. [1]FM=fish meal-based reference diet, S20=diet with 20% replacement of fish meal with *Saccharomyces cerevisiae*, S40=diet with 40% replacement of fish meal with *S. cerevisiae,* S60=diet with 60% replacement of fish meal with *S. cerevisiae,* S60-Met=diet with 60% replacement of fish meal with *S. cerevisiae* and no added crystalline methionine, W20=diet with 20% replacement of fish meal with *Wickerhamomyces anomalus/S. cerevisiae* mix, W40=diet with 40% replacement of fish meal with *W. anomalus/S. cerevisiae* mix, W60=diet with 60% replacement of fish meal with *W. anomalus/S. cerevisiae* mix.

Drum feeders were replaced with manually loaded band feeders after the second weighing and the subsequent growth of fish fed high yeast levels was the highest recorded in the experiment, quite possibly due to compensatory growth (Figure 9).

Previous studies, such as that by de la Higuera *et al.* (1981), have shown that total replacement of fish meal in diets for rainbow trout with pure *W. anomalus* cause decreased feed intake and growth. Gause and Trushenski (2011) demonstrated that complete replacement of fish meal with ethanol yeast *S. cerevisiae* impairs both growth performance and feed conversion ratio. This led those authors to recommend that yeast should not be used as a single protein source in fish diets, but rather in combination with fish meal.

Hauptman *et al.* (2014) found that replacing more than 37.5 % of fish meal with grain distillers dried yeast negatively affects the overall performance in rainbow trout. This inclusion level corresponds to 11.2% of the diet. However, in Papers II and III considerably higher inclusion levels of yeasts were used without negative effects on growth performance.

Table 7. *Growth performance and relative nutrient retention of Arctic charr in Paper II. FW = final body weight, SGR = specific growth rate, WG = weight gain, FCR = feed conversion ratio s.e. = standard error*

	Diet[1]						
	REF	**MYE**	**ISC**	**ESC**	**RHO**	**s.e.**	**P-value**
FW (g) [2]	133.3[a]	126.4[ab]	125.6[ab]	117.9[b]	118.5[b]	1.89	0.001
SGR (% day^{-1})[2]	1.08[a]	1.02[ab]	1.04[ab]	0.95[b]	0.97[b]	0.01	0.001
WG (%)[2]	179.4[a]	165.7[ab]	166.7[ab]	149.7[b]	149.7[b]	3.95	0.001
FCR (g g^{-1})	0.89	0.93	0.95	0.98	1.01	0.03	0.064
Nutrient retention (%)							
Protein (N x 6.25)	43.5[a]	39.5[ab]	40.2[ab]	38.1[b]	39.3[ab]	1.05	0.041
Total amino acids	44.4[a]	42.9[a]	36.8[ab]	33.0[b]	38.8[ab]	1.92	0.012
Energy	43.6	42.3	40.7	39.8	38.2	1.24	0.075

[1]REF = reference diet, MYE = diet with blue mussel (*M. edulis*), ISC = diet with intact yeast (*S. cerevisiae*), ESC = diet with extracted yeast (ESC), RHO = diet with filamentous fungi *R. oryzae*.
[2]When analysing SW, FW, WG and SGR, n=145 for REF; n= 145 for MYE; n=140 for ISC; n=141 for ESC; n=142 for RHO.
Values within rows with different superscripts are significantly different (P<0.05).

Table 8. *Growth performance, daily feed intake and nutrient retention of rainbow trout in Paper III. FW = final body weight, SGR = specific growth rate, WG = weight gain, FCR = feed conversion ratio, DFI = daily feed intake. Data presented are least square means. s.e. = pooled standard error.*

	Diet[1]									
	FM	**S20**	**S40**	**S60**	**S60-Met**	**W20**	**W40**	**W60**	**s.e.**	**P-value**
FW (g) [2]	374.2	355.30	357.11	290.27	304.76	348.49	323.96	289.40	13.17	0.06
SGR (% day^{-1})[2]	1.33^{a}	1.28^{a}	1.27^{ab}	1.02^{ab}	1.10^{ab}	1.28^{a}	1.12^{ab}	0.99^{b}	0.05	0.04
WG (%)[2]	153.38^{*}	144.83^{*}	143.47	103.86	116.99	145.07^{*}	119.06	100.16^{*}	8.64	0.06
FCR (g g^{-1})[2]	0.92	0.91	1.00	0.94	0.97	0.89	0.94	0.97	0.07	0.64
DFI (% of bw)[2]	1.67^{a}	1.52^{ab}	1.58^{ab}	0.87^{b}	1.20^{ab}	1.50^{ab}	1.24^{ab}	1.38^{ab}	0.15	0.04
Nutrient retention (%)										
Protein (Nx6.25)[2]	46.77	48.20	42.71	48.08	44.92	49.30	45.18	43.44	2.24	0.17
Crude lipids[2]	89.77	87.42	79.42	74.65	81.74	88.08	78.52	82.07	4.36	0.55
Energy[2]	45.3	47.3	41.1	39.2	40.4	46.2	41.0	38.0	3.07	0.73

[1]FM=fish meal-based reference diet, S20=diet with 20% replacement of fish meal with *Saccharomyces cerevisiae*, S40=diet with 40% replacement of fish meal with *S. cerevisiae,* S60=diet with 60% replacement of fish meal with *S. cerevisiae,* S60-Met=diet with 60% replacement of fish meal with *S. cerevisiae* and no added crystalline methionine, W20=diet with 20% replacement of fish meal with *Wickerhamomyces anomalus/S. cerevisiae* mix, W40=diet with 40% replacement of fish meal with *W. anomalus/S. cerevisiae* mix, W60=diet with 60% replacement of fish meal with *W. anomalus/S. cerevisiae* mix.
[2] n= 3. Values within rows with different superscripts are significantly different ($P<0.05$).

5.4 Active transport and intestinal barrier function

It has been shown that the yeast cell wall compounds mannoproteins, β-glucans and chitin can have immuno-modulating effects, enhance intestinal barrier function and increase growth performance in fish (Navarrete & Tovar-Ramirez, 2014; Torrecillas *et al.*, 2014; Torrecillas *et al.*, 2011; Refstie *et al.*, 2010). Chitin and chitosan, which are present in cell walls of *R. oryzae*, have also been shown to have positive effects on immune status in fish (Harikrishnan *et al.*, 2012; Esteban *et al.*, 2001).

In Paper II, diets with intact *S. cerevisiae* and *R. oryzae* caused increased paracellular permeability. Earlier studies using *R. oryzae* in diets for Arctic charr reported impaired intestinal barrier function (Abro *et al.*, 2014). Such changes can often lead to increased disease susceptibility and intestinal inflammation (Segner *et al.*, 2012), but at the same time to positive effects for the fish through immunostimulation. Niklasson *et al.* (2011) and Torrecillas *et al.* (2011) concluded that increased paracellular permeability, i.e. leakage, can increase the flow of antigens through the epithelium and consequently trigger the immune system to react and decrease the translocation of pathogenic bacteria. As the presence of intestinal inflammation in Paper II could not be assessed, it remains unclear whether increased intestinal permeability caused stimulation or impairment of intestinal barrier function.

5.5 Post-prandial amino acid profiles and whole blood parameters

In Paper IV, the effect of yeast inclusion on post-prandial changes in plasma free amino acids and whole blood parameters was monitored in dorsal aorta (DA) cannulated, voluntarily feeding rainbow trout. This study was one of the first to be successful in maintaining voluntary feeding in rainbow trout equipped with DA cannula.

The significant increases observed in pH, TCO_2, $HCO_3$and BE in the whole blood of fish fed a diet with intact *S. cerevisiae* and a diet with *W. anomalus/S. cerevisiae* mix may indicate acute alkalosis. Since no changes were found in blood PCO_2 with respect to dietary treatment or sampling time, alkalosis is most likely not of respiratory, but of metabolic origin. One possible reason could be low content of Na and high content of K in the yeast diets (Table 5). Goss and Perry (1994) demonstrated metabolic alkalosis in rainbow trout by intra-arterially infusing $NaHCO_3$ and reported complete recovery within 12 hours via increased branchial Cl^- uptake and H^+ excretion. In the

long run alkalosis could induce increased energy expenditure due to increased blood pH regulation and thus influence the growth performance. Therefore, it would be beneficial to balance the mineral content of yeast in order to prevent alkalosis in fish.

Fish in the stress test displayed decreases in blood pH, TCO_2, HCO_3 and BE levels, reaching a 3 hour low. This may indicate acute respiratory acidosis caused by gills hyperventilation in response to stress and possible anaerobic glycolysis. In response, the levels of pH and gases increased over initial levels and returned to basal levels at 12 hours. Similar observations of elevated post-stress levels of pH and blood gases in rainbow trout were reported previously by Turner *et al.* (1983).

Glucose concentration increased later (12 hours) in fish fed diets with yeast compared with fish fed the reference fish meal-based diet (6 hours). This was most likely due to diet composition as the reference diet contained higher levels of starch and cellulose, which can affect blood glucose levels and postprandial uptake in rainbow trout (del sol Novoa *et al.*, 2004; Kaushik *et al.*, 1989; Bergot, 1979).

Post-prandial Hct, Lct and Hb values decreased for all dietary treatments indicating haemodilution as an effect of frequent sampling and inability of the haematopoietic system to replenish the erythrocytes between samplings (Bry & Zohar, 1980; Soivio *et al.*, 1975). The continual reduction in RBC also confirms this sampling effect.

There was a significant effect of diet on whole blood MCH values. The MCH level was significantly higher for fish fed the diet with *W. anomalus/S. cerevisiae* mix than for fish fed the reference diet, which may indicate hyperchromic anaemia. In addition, fish fed the diet with intact *S. cerevisiae* displayed a tendency for higher MCH values compared with fish fed the reference diet. Furthermore, in fish fed the diet with *W. anomalus/S. cerevisiae*, post-prandial MCH levels showed a decreasing trend. Similarly, Sánchez-Muniz *et al.* (1982) used yeast *W. anomalus* as the only protein source in diets for rainbow trout and revealed that the MCH values dropped post feeding, an effect which they attributed to oxidative stress caused by hydrogen peroxide, a product of nucleic acid degradation in yeast. In addition, they found MCH values to be lower in fish fed the yeast diet than in fish fed the reference diet, indicating hypochromic anaemia in the former. This difference in the observations is likely the result of the feed formulation and the trial length. Sánchez-Muniz *et al.* (1982) used diets containing 81% of yeast inclusion and fed the fish to satiation for a period of 3 weeks whereas levels in paper IV varied from 32-35% and the fish were fed for one week.

Due to the lower methionine content, diets with yeasts were supplemented with crystalline methionine in Papers III and IV. Analysis of postprandial levels of plasma free amino acids revealed differences between dietary treatments with respect to methionine levels, which were higher in fish fed diets with yeasts than in fish fed the reference diet. Other studies have also reported higher maximum levels in rainbow trout diets supplemented with crystalline amino acids (Rolland *et al.*, 2015; Schuhmacher *et al.*, 1997; Cowey & Walton, 1988; Yamada *et al.*, 1981). Furthermore, methionine levels in Paper IV peaked at 6 hours for all dietary treatments.

Schuhmacher *et al.* (1997) showed that crystalline amino acids in diets for rainbow trout were absorbed more rapidly than amino acids from whole proteins and argued that this could cause an imbalance in timing of amino acid absorption. This was not observed in Paper IV, although there is a possibility of a missed peak between the sampling points. However, when present in surplus, methionine can be metabolised into sarcosine (Benevenga, 1974). The sarcosine plasma levels were consistently higher in fish fed both diets with yeasts in Paper IV, which may indicate that the methionine was simply metabolised into sarcosine.

Hydroxy-lysine-2 and hydroxy-proline, derivatives of lysine and proline, were present in lower levels in fish fed diets with yeast than in fish fed the reference diet. However, lysine and proline content did not vary between the diets, indicating that differences in post-prandial levels of hydroxy-lysine-2 and hydroxy-lysine were not caused by catabolism of lysine and proline. Hydroxy-lysine-2 and hydroxy-proline, together with glycine and other amino acids, form collagen, a structural protein present in connective tissue in animals (Cassab & Varner, 1988; Balian & Bowes, 1977). The higher levels of these amino acids observed in fish fed the reference diet were most likely a result of higher collagen content in fish meal than yeasts.

6 Concluding remarks

- Based on the results presented in this thesis, it can be concluded that 40% (crude protein basis) of fish meal in the diet of Arctic charr can be replaced with blue mussel (*Mytilus edulis*) or intact baker's yeast (*Saccharomyces cerevisiae*) in diets for Arctic charr without negative effects on growth performance and nutrient retention. However, use of the filamentous fungus (*Rhizopus oryzae*) and extracted *S. cerevisiae* to replace fish meal in diets for Arctic charr did not yield satisfactory results in terms of growth performance.

- Experiment with rainbow trout showed that both intact baker's yeast and a combination of the yeasts *Wickerhamomyces anomalus* and *S. cerevisae* can be used to replace 40% of fish meal on a digestible protein basis without causing impaired growth or nutrient retention.

- The Eurasian perch may have a higher capacity for digesting *R. orizae* and intact *S. cerevisiae* and these ingredients could possibly be included in higher amounts in diets for perch and other species with similar digestive capacity.

- Chemical analysis of test ingredients revealed slight methionine deficiency in *R. oryzae* and intact *S. cerevisae*, but fish growth data indicated that methionine supplementation may not be necessary when such high yeast levels are used.

- Although methionine supplementation did not seem to affect growth performance in fish fed diets with high yeast inclusion, diets containing fungal protein sources were regularly low in methionine. Thus methionine supplementation should still be considered in diets containing these protein sources.

- Intestinal barrier function in Arctic charr seemed to be affected by inclusion of *R. oryzae* and intact *S. cerevisiae* in the diet. Presence of intact *S. cerevisiae* and *R. oryzae* in the diet caused increased leakage in the intestine of Arctic charr, most likely as an effect of bioactive cell wall components on paracellular permeability.

- It was found that high inclusion rates of *S. cerevisiae* and *W. anomalus/S. cerevisiae* can affect whole blood chemistry and plasma amino acid profiles in rainbow trout. Yeast inclusion in rainbow trout diets seemed to trigger temporary blood alkalosis, which was related to low sodium and high potassium content in yeasts. This could affect the health status and performance of rainbow trout fed diets with yeasts and efforts should be made to balance the mineral content of yeasts in diets for rainbow trout.

- The use of the dorsal aorta cannulation technique on voluntarily feeding rainbow trout proved to be a valuable tool for measuring post-prandial metabolic changes in blood parameters and plasma amino acids.

7 Future perspectives

Certain concerns in relation to some of the test ingredients used in this thesis have been revealed that could, if resolved, increase the quality and practical value of these protein sources.

Overall, all fungal protein sources used in this thesis have been lower on protein content than the fish meal, which may be one of the limiting factors to their use in commercial diets. Further efforts should focus towards selection and optimization of substrates and production conditions for growth of fungi in order to improve their protein content and amino acid profile.

Extrusion appears to improve the utilisation of feed containing intact yeasts when compared to previous studies using pelleting as the choice of production method. Nevertheless, the effects of extrusion on utilisation of yeasts in diets for fish and the underlying technical processes were not studied in this thesis and should be examined in more details.

Decreased digestibility of intact *S. cerevisiae* observed in papers I and II has been attributed to the presence of cell walls. This was supported by higher digestibility of extracted *S. cerevisiae*. However, although autolysis as a method of cell wall disruption used in the production of extracted *S. cerevisiae* yeast improves digestibility in diets to charr, it decreases its nutritional value. Further efforts should be placed on researching new ways for cell wall disruption while maintaining the nutritive integrity of yeasts. In prospect, diets containing whole yeast could be supplemented or treated with appropriate enzymes during or after the feed production process.

Differences in digestion of intact *S. cerevisiae* and *R. oryzae* between Arctic charr and Eurasian perch indicate that these protein sources may be more suitable in diets for some non-salmonid species. However, this should be confirmed by testing on other species.

Feed containing extracted *S. cerevisiae* and *R. oryzae* in paper I, as well as feed with high inclusion of intact *S. cerevisiae* and *W. anomalus/S. cerevisiae* mix in paper III, reveals issues in relation to pellet expansion and consequent oil absorption. In order to improve the pellet quality, further work should focus on examining technical properties of these ingredients in combination with optimizing the extrusion configuration.

The effects of fungi, yeasts and blue mussel inclusion in feed on the quality of the final product, i.e. fish fillet haven't been studied in this thesis. However, parallel work at the same University indicate that the sensory quality is similar to that of fish fed fish meal base diet This is an important aspect if these protein sources are to be used commercially and should be examined further.

The effect of whole yeast inclusion in fish diets on indigenous intestinal microbiota is relatively unknown and is currently explored in long term studies at our University, in order to determine potential effects on the nutrient utilisation and health status in fish. Furthermore, efforts should be directed to examining the effects of yeast containing diets on the intestinal inflammatory status.

Increased digestibility of phosphorus in the diets with *W. anomalus/S. cerevisiae* mix may be an effect of phytase activity by *W. anomalus* in the feed, however not enough evidence exist to support this observation. Thus, further research should be conducted in order to assess the phytase activity in *W. anomalus* when included in diets for fish.

In addition, the use of multi-disciplinary approach in future studies dealing with novel proteins sources for fish may offer more insights into possibilities and limitations of these sources in diets to fish, thus contribute to their optimization.

8 Svensk sammanfattning

I avhandlingen har svamp och musslor utvärderats som proteinkälla i foder till abborre (*Perca fluviatilis*), röding (*Salvelinus Alpinus*) och regnbågslax (*Onchorhynchus mykiss*). Fodrets inverkan på tillväxt, näringsutnyttjande, effekt på tarmbarriären och blodplasmaprofilen av aminosyror och andra blodparametrar efter födointag har studerats.

I försöken med röding påvisades att den skenbara smältbarheten av torrsubstans, summan av aminosyror och bruttoenergi minskade med ökad inblandning av intakt *Saccharomyces cerevisiae* i fodret. Däremot kunde inga skillnader i skenbar smältbarhet påvisas mellan de olika fodermedlen i studier med abborre. Dessa resultat ger en indikation om att abborre har en högre digestionskapacitet för att utnyttja *S. cerevisiae* jämfört med röding, som påverkats negativt av dieter innehållande intakta jäst-cellväggar.

Tillväxt, proteinretention och totala aminosyror påverkades inte hos röding som utfodrats med foder innehållande intakt *S. cerevisiae* och *M. edulis*, vilket visar att upp till 40 % av fiskmjöl kan bytas ut mot jäst och musselmjöl i foder till röding. Däremot påvisades att foder innehållande *S. cerevisiae* och *R. oryzae* hade en högre skenbar permeabilitet på tarmbarriären jämfört med referensfodret som var baserat på fiskmjöl. Det transepiteliala motståndet i den proximala tarmen påverkades inte av någon av de testade dieterna.

Hos regnbågslax påvisades ingen effekt på foderomvandling och tillväxt när fiskmjöl ersatts med motsvarande mängd av intakt *S. cerevisiae* eller *Wickerhamomyces anomalus / S. cerevisiae* mix. Den skenbara smältbarheten av råprotein var opåverkad när 20 % av fiskmjöl ersattes med jästmix. Resultaten från denna studie överensstämmer med den studie som gjordes på röding och som visade att 40 % av fiskmjöl kan bytas ut mot intakt *S. cerevisiae* utan att äventyra tillväxten hos fiskarna.

Resultaten av olika blodparametrar hos regnbågslax visade en signifikant ökning av pH, TCO_2, HCO_3 i helblod och basöverskott hos fisk som utfodrats med ”jästfoder” jämfört med kontrollfoder. De erhållna resultaten tyder på en akut metabolisk alkalos beroende på skillnader i mineralinnehåll hos de olika dieterna. Aminosyraprofilen i blodet visade att metionin, hydroxy-lysin-2, 3-metyl-histidin och hydroxy-prolin skilde sig signifikant mellan fisk som

utfodrats med ”jästfoder” respektive kontrollfoder. En trolig orsak är olika innehållsmängd av kristallint metionin och kollagen i jäst- och fiskmjölsbaserade dieter.

References

Abro, R., Sundell, K., Sandblom, E., Sundh, H., Brännäs, E., Kiessling, A., Lindberg, J.E. & Lundh, T. (2014). Evaluation of chitinolytic activities and membrane integrity in gut tissues of Arctic charr (Salvelinus alpinus) fed fish meal and zygomycete biomass. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* 175(0), pp. 1-8.

Ambardekar, A.A., Reigh, R.C. & Williams, M.B. (2009). Absorption of amino acids from intact dietary proteins and purified amino acid supplements follows different time-courses in channel catfish (Ictalurus punctatus). *Aquaculture,* 291(3–4), pp. 179-187.

Andersen, Ø., Aas, T.s., Skugor, S., Takle, H., van Nes, S., Grisdale-Helland, B., Helland, S.J. & Terjesen, B.F. (2006). Purine-induced expression of urate oxidase and enzyme activity in Atlantic salmon (Salmo salar). *FEBS Journal,* 273(13), pp. 2839-2850.

Balian, G. & Bowes, J.H. (1977). The structure and properties of collagen In: Ward, A.G. & Courts, A. (eds) *The science and technology of gelatin*. London: Academic press, pp. 1-31.

Ballantyne, J.S. (2001). *Amino acid metabolism*. (Fish Physiology 20). San Diego, CA: Academic Press.

Barrows, F.T., Stone, D.A.J. & Hardy, R.W. (2007). The effects of extrusion conditions on the nutritional value of soybean meal for rainbow trout (Oncorhynchus mykiss). *Aquaculture,* 265(1–4), pp. 244-252.

Benevenga, N.J. (1974). Toxicities of methionine and other amino acids. *Journal of Agricultural and Food Chemistry,* 22(1), pp. 2-9.

Berge, G.M. & Austreng, E. (1989). Blue mussel in feed for rainbow trout. *Aquaculture,* 81(1), pp. 79-90.

Bergot, F. (1979). Effects of dietary carbohydrates and of their mode of distribution on glycaemia in rainbow trout (Salmo gairdneri richardson). *Comparative Biochemistry and Physiology Part A: Physiology,* 64(4), pp. 543-547.

Booth, M A., Allan, G L., Evans, A J. & Gleeson, V P. (2002). Effects of steam pelleting or extrusion on digestibility and performance of silver perch Bidyanus bidyanus. *Aquaculture Research,* 33(14), pp. 1163-1173.

Brown, L.R. (2012). *Full Planet, Empty Plates: The New Geopolitics of Food Scarcity*: W. W. Norton. Available from: http://books.google.se/books?id=yQWj1E9DLt0C.

Bry, C. & Zohar, Y. (1980). Dorsal aorta catheterization in rainbow trout (Salmo gairdneri). II. Glucocorticoid levels, hematological data and resumption of feeding for five days after surgery. *Reprod Nutr Dev,* 20(6), pp. 1825-34.

Brännäs, E. & Linnér, J. (2000). Growth effects in Arctic charr reared in cold water: Feed frequency, access to bottom feeding and stocking density. *Aquaculture International,* 8(5), pp. 381-389.

Bureau, D.P., Harris, A.M. & Cho, C.Y. (1999). Apparent digestibility of rendered animal protein ingredients for rainbow trout (Oncorhynchus mykiss). *Aquaculture,* 180(3–4), pp. 345-358.

Carter, C., Houlihan, D., Kiessling, A., Médale, F. & Jobling, M. (2007). Physiological Effects of Feeding. In: *Food Intake in Fish* Blackwell Science Ltd, pp. 297-331. Available from: http://dx.doi.org/10.1002/9780470999516.ch13.

Cassab, G.I. & Varner, J.E. (1988). Cell Wall Proteins. *Annual Review of Plant Physiology and Plant Molecular Biology,* 39(1), pp. 321-353.

Chikwati, E.M. (2013). *Diet-induced physiological and pathophysiological responses in the intestine of Atlantic salmon (Salmo salar L.) : effects of plant protein ingredients and antinutritional factors.* Diss.: Norwegian School of Veterinary Science

Cho, C.Y. & Kaushik, S.J. (1990). Nutritional energetics in fish: energy and protein utilization in rainbow trout (Salmo gairdneri). *World Rev Nutr Diet,* 61, pp. 132-72.

Cho, C.Y. & Slinger, S.J. (1979). Apparent Digestibility Measurement in Feedstuffs for Rainbow Trout. In, pp. P239-248.

Cho, C.Y., Slinger, S.J. & Bayley, H.S. (1982). Bioenergetics of salmonid fishes: Energy intake, expenditure and productivity. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry,* 73(1), pp. 25-41.

Conte, F.P., Wagner, H.H. & Harris, T.O. (1963). Measurement of blood volume in the fish (Salmo gairdneri gairdneri). *American Journal of Physiology -- Legacy Content,* 205(3), pp. 533-540.

Cowey, C.B. & Walton, M.J. (1988). Studies on the uptake of (14C) amino acids derived from both dietary (14C) protein and dietary (14C) amino acids by rainbow trout, Salmo gairdneri Richardson. *Journal of Fish Biology,* 33(2), pp. 293-305.

Dalsgaard, J., Lund, I., Thorarinsdottir, R., Drengstig, A., Arvonen, K. & Pedersen, P.B. (2013). Farming different species in RAS in Nordic countries: Current status and future perspectives. *Aquacultural Engineering,* 53(0), pp. 2-13.

de la Higuera, M., Sánchez-Muniz, F.J., Mataix, F.J. & Varela, G. (1981). Nitrogen utilization by rainbow trout (Salmo gairdneri) fed on the yeast Hansenula anomala. *Comparative Biochemistry and Physiology Part A: Physiology,* 69(3), pp. 583-586.

del sol Novoa, M., Capilla, E., Rojas, P., Baro, J., Gutierrez, J. & Navarro, I. (2004). Glucagon and insulin response to dietary carbohydrate in rainbow trout (Oncorhynchus mykiss). *Gen Comp Endocrinol,* 139(1), pp. 48-54.

Djordjevic, B., Kristensen, T., Øverli, Ø., Rosseland, B.O. & Kiessling, A. (2012). Effect of nutritional status and sampling intensity on recovery after dorsal aorta cannulation in free-swimming Atlantic salmon (Salmo salar L.). *Fish Physiology and Biochemistry,* 38(1), pp. 259-272.

Eddy, F.B. & Fraser, J.E. (1982). Sialic acid and mucus production in rainbow trout (Salmo gairdneri Richardson) in response to zinc and seawater. *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology,* 73(2), pp. 357-359.

Edebo, L. (2008). Zygomycetes for fish feed. Google Patents.

Eliason, E.J., Djordjevic, B., Trattner, S., Pickova, J., Karlsson, A., Farrell, A.P. & Kiessling, A.K. (2010). The effect of hepatic passage on postprandial plasma lipid profile of rainbow trout (Oncorhynchus mykiss) after a single meal. *Aquaculture Nutrition,* 16(5), pp. 536-543.

Espe, M., Lied, E. & Torrissen, K.R. (1993). Changes in plasma and muscle free amino acids in atlantic salmon (Salmo salar) during absorption of diets containing different amounts of hydrolysed cod muscle protein. *Comparative Biochemistry and Physiology Part A: Physiology,* 105(3), pp. 555-562.

Esteban, M.A., Cuesta, A., Ortuño, J. & Meseguer, J. (2001). Immunomodulatory effects of dietary intake of chitin on gilthead seabream (Sparus aurata L.) innate immune system. *Fish & Shellfish Immunology,* 11(4), pp. 303-315.

FAO (2012). *The State of World Fisheries and Aquaculture*. Rome, Italy: United Nations Food and Agriculture Organization.

FAO (2014). *The State of World Fisheries and Aquaculture*. Rome, Italy: Fisheries and Aquaculture Department

Fauconneau, B. & Arnal, M. (1985). Leucine metabolism in trout (Salmo gairdnerii R.). Influence of temperature. *Comparative Biochemistry and Physiology Part A: Physiology,* 82(2), pp. 435-445.

FEFAC (2013). *EU Reauthorisation of non-ruminant processed animal proteins for fish feed*. Brussels.

Ferreira, J., Lennartsson, P., Niklasson, C., Lundin, M., Edebo, L. & Taherzadeh, M. (2012). Spent Sulphite Liquor for Cultivation of an Edible Rhizopus Sp. *BioResources, North America,* 7.

Fredlund, E., Druvefors, U.A., Olstorpe, M.N., Passoth, V. & Schnurer, J. (2004). Influence of ethyl acetate production and ploidy on the anti-mould activity of Pichia anomala. *FEMS Microbiol Lett,* 238(1), pp. 133-7.

Gatesoupe, F.J. (2007). Live yeasts in the gut: Natural occurrence, dietary introduction, and their effects on fish health and development. *Aquaculture,* 267(1–4), pp. 20-30.

Gatlin, D.M., Barrows, F.T., Brown, P., Dabrowski, K., Gaylord, T.G., Hardy, R.W., Herman, E., Hu, G., Krogdahl, Å., Nelson, R., Overturf, K., Rust, M., Sealey, W., Skonberg, D., J Souza, E., Stone, D., Wilson, R. &

Wurtele, E. (2007). Expanding the utilization of sustainable plant products in aquafeeds: a review. *Aquaculture Research,* 38(6), pp. 551-579.
Gause, B. & Trushenski, J. (2011). Replacement of Fish Meal with Ethanol Yeast in the Diets of Sunshine Bass. *North American Journal of Aquaculture,* 73(2), pp. 97-103.
Glencross, B., Hawkins, W. & Curnow, J. (2004). Nutritional assessment of Australian canola meals. I. Evaluation of canola oil extraction method and meal processing conditions on the digestible value of canola meals fed to the red seabream (Pagrus auratus, Paulin). *Aquaculture Research,* 35(1), pp. 15-24.
Glencross, B.D., Booth, M. & Allan, G.L. (2007). A feed is only as good as its ingredients – a review of ingredient evaluation strategies for aquaculture feeds. *Aquaculture Nutrition,* 13(1), pp. 17-34.
Goss, G.G. & Perry, S.F. (1994). Different Mechanisms of Acid-Base Regulation in Rainbow Trout (Oncorhynchus mykiss) and American Eel (Anguilla rostrata) during $NaHCO_3$ Infusion. *Physiological Zoology,* 67(2), pp. 381-406.
Hajen, W.E., Beames, R.M., Higgs, D.A. & Dosanjh, B.S. (1993). Digestibility of various feedstuffs by post-juvenile chinook salmon (Oncorhynchus tshawytscha) in sea water. 1. Validation of technique. *Aquaculture,* 112(4), pp. 321-332.
Harikrishnan, R., Kim, J.-S., Balasundaram, C. & Heo, M.-S. (2012). Immunomodulatory effects of chitin and chitosan enriched diets in Epinephelus bruneus against Vibrio alginolyticus infection. *Aquaculture,* 326–329(0), pp. 46-52.
Hauptman, B.S., Barrows, F.T., Block, S.S., Gibson Gaylord, T., Paterson, J.A., Rawles, S.D. & Sealey, W.M. (2014). Evaluation of grain distillers dried yeast as a fish meal substitute in practical-type diets of juvenile rainbow trout, Oncorhynchus mykiss. *Aquaculture,* 432(0), pp. 7-14.
Helland, S.J., Grisdale-Helland, B. & Nerland, S. (1996). A simple method for the measurement of daily feed intake of groups of fish in tanks. *Aquaculture,* 139(1–2), pp. 157-163.
HLPE (2014). *Sustainable fisheries and aquaculture for food security and nutrition.* . Rome: High Level Panel of Experts on Food Security and Nutrition of the Committee on World Food Security.
Hooft, J.M., Elmor, A.E.H.I., Encarnação, P. & Bureau, D.P. (2011). Rainbow trout (Oncorhynchus mykiss) is extremely sensitive to the feed-borne Fusarium mycotoxin deoxynivalenol (DON). *Aquaculture,* 311(1–4), pp. 224-232.
IFFO (2015). *IFFO update* 226): International Fish Meal and Fish Oil Organisation.
Izzo, A.A., Gaginella, T.S., Mascolo, N. & Capasso, F. (1994). Nitric oxide as a mediator of the laxative action of magnesium sulphate. *Br J Pharmacol,* 113(1), pp. 228-32.
Jobling, M., Jørgensen, E.H., Arnesen, A.M. & Ringø, E. (1993). Feeding, growth and environmental requirements of Arctic charr: a review of aquaculture potential. *Aquaculture International,* 1(1), pp. 20-46.

Jutfelt, F. (2011). Integrated function and control of the gut | Barrier Function of the Gut. In: Farrell, A.P. (ed. *Encyclopedia of Fish Physiology*. San Diego: Academic Press, pp. 1322-1331. Available from: http://www.sciencedirect.com/science/article/pii/B978012374553800068X.

Karlsson, A., Eliason, E.J., Mydland, L.T., Farrell, A.P. & Kiessling, A. (2006). Postprandial changes in plasma free amino acid levels obtained simultaneously from the hepatic portal vein and the dorsal aorta in rainbow trout (Oncorhynchus mykiss). *Journal of Experimental Biology,* 209(24), pp. 4885-4894.

Kaushik, S.J., Medale, F., Fauconneau, B. & Blanc, D. (1989). Effect of digestible carbohydrates on protein/energy utilization and on glucose metabolism in rainbow trout (Salmo gairdneri R.). *Aquaculture,* 79(1–4), pp. 63-74.

Kaushik, S.J. & Seiliez, I. (2010). Protein and amino acid nutrition and metabolism in fish: current knowledge and future needs. *Aquaculture Research,* 41(3), pp. 322-332.

Khan, F.R. & McGeer, J.C. (2013). Zn-stimulated mucus secretion in the rainbow trout (Oncorhynchus mykiss) intestine inhibits Cd accumulation and Cd-induced lipid peroxidation. *Aquatic Toxicology,* 142–143(0), pp. 17-25.

Kiessling, A. (2009). Feed-the key to sustainable fish farming. In: Ackefors, H., Cullberg, M., Wramner, P. Sundberg (ed. *KSLA- Fish, Trade and Development*, pp. 303-322.

Kiessling, A. & Askbrandt, S. (1993). Nutritive value of two bacterial strains of single-cell protein for rainbow trout (Oncorhynchus mykiss). *Aquaculture,* 109(2), pp. 119-130.

Kiessling, A., Dosanjh, B., Higgs, D., Deacon, G. & Rowshandeli, N. (1995). Dorsal aorta cannulation: a method to monitor changes in blood levels of astaxanthin in voluntarily feeding Atlantic salmon, Salmo salar L. *Aquaculture Nutrition,* 1(1), pp. 43-50.

Kiessling, A., Kiessling, K.H., Storebakken, T. & Åsgård, T. (1991). Changes in the structure and function of the epaxial muscle of rainbow trout (Oncorhynchus mykiss) in relation to ration and age: II. Activity of key enzymes in energy metabolism. *Aquaculture,* 93(4), pp. 357-372.

Kinsella, J.E., German, B. & Shetty, J. (1985). Uricase from fish liver: Isolation and some properties. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry,* 82(4), pp. 621-624.

Knudsen, D., Jutfelt, F., Sundh, H., Sundell, K., Koppe, W. & Frokiaer, H. (2008). Dietary soya saponins increase gut permeability and play a key role in the onset of soyabean-induced enteritis in Atlantic salmon (Salmo salar L.). *Br J Nutr,* 100(1), pp. 120-9.

Krogdahl, Å., Penn, M., Thorsen, J., Refstie, S. & Bakke, A.M. (2010). Important antinutrients in plant feedstuffs for aquaculture: an update on recent findings regarding responses in salmonids. *Aquaculture Research,* 41(3), pp. 333-344.

Kuhad, R.C., Singh, A., Tripathi, K.K., Saxena, R.K. & Eriksson, K.-E.L. (1997). Microorganisms as an Alternative Source of Protein. *Nutrition Reviews,* 55(3), pp. 65-75.

Langeland, M., Lindberg, J.E. & Lundh, T. (2013). Digestive Enzyme Activity in Eurasian Perch (*Perca fluviatilis*) and Arctic charr (*Salvelinus Alpinus*). *Journal of Aquaculture Research and Development,* 5(208).

Langeland, M., Vidakovic, A., Vielma, J., Lindberg, J.E., Kiessling, A. & Lundh, T. (2014). Digestibility of microbial and mussel meal for Arctic charr (Salvelinus alpinus) and Eurasian perch (Perca fluviatilis). *Aquaculture Nutrition*, pp. n/a-n/a.

Li, P., Mai, K., Trushenski, J. & Wu, G. (2009). New developments in fish amino acid nutrition: towards functional and environmentally oriented aquafeeds. *Amino Acids,* 37(1), pp. 43-53.

Lindahl, O., Hart, R., Hernroth, B., Kollberg, S., Loo, L.-O., Olrog, L., Rehnstam-Holm, A.-S., Svensson, J., Svensson, S. & Syversen, U. (2005). Improving Marine Water Quality by Mussel Farming: A Profitable Solution for Swedish Society. *AMBIO: A Journal of the Human Environment,* 34(2), pp. 131-138.

Llames, C.R. & Fontaine, J. (1994). Determination of Amino-Acids in Feeds - Collaborative Study. *J AOAC Int,* 77(6), pp. 1362-1402.

Lo, W.-Y., Chang, C.-F. & Song, Y.-L. (2003). Evaluation of dorsal aorta cannulation for immunological studies of grouper (Epinephelus malabaricus). *Fish & Shellfish Immunology,* 14(4), pp. 289-303.

Lupatsch, I., Kissil, G.W. & Sklan, D. (2003). Comparison of energy and protein efficiency among three fish species gilthead sea bream (Sparus aurata), European sea bass (Dicentrarchus labrax) and white grouper (Epinephelus aeneus): energy expenditure for protein and lipid deposition. *Aquaculture,* 225(1–4), pp. 175-189.

Mertens, D.R. (2002). Gravimetric determination of amylase-treated neutral detergent fiber in feeds with refluxing in beakers or crucibles: collaborative study. *J AOAC Int,* 85(6), pp. 1217-40.

Murray, A.P. & Marchant, R. (1986). Nitrogen utilization in rainbow trout fingerlings (Salmo gairdneri Richardson) fed mixed microbial biomass. *Aquaculture,* 54(4), pp. 263-275.

Mydland, L.T., Djordjevic, B., Øverland, M., Skrede, A., Zimonja, T. & Kiessling, A. (2009). Alternative protein sources modulate postprandial plasma amino acids profiles in Atlantic

salmon (Salmo salar L.). In: Djordjevic, B. (ed. *Physiological and genomic responses in salmonids with special emphasis on welfare and novel feed ingredients*. Ås: Norwegian University of Life Sciences.

Mydland, L.T., Landsverk, T., Zimonja, T., Storebakken, T., Edebo, L. & Kiessling, A. (2007). Mycelium biomass from fungi (*Rhizopus oryzae*) grown on spent sulphite liquor from paper pilp as a protein source in diets for Rainow trout (O*nchorhynchus mykiss*). *Aquaculture Europe 2007, Book of abstracts.* Istanbul, Turkey, pp. 376-376.

Nagel, F., von Danwitz, A., Schlachter, M., Kroeckel, S., Wagner, C. & Schulz, C. (2014). Blue mussel meal as feed attractant in rapeseed protein-based diets for turbot (Psetta maxima L.). *Aquaculture Research,* 45(12), pp. 1964-1978.

Nasseri, A.T., Rasoul-Amini, S., Morowvat, M.H. & Ghasemi, Y. (2011). Single Cell Protein: Production and Process. *American Journal of Food Technology,* 6(2), pp. 103-116.

Navarrete, P. & Tovar-Ramirez, D. (2014). *Use of Yeasts as Probiotics in Fish Aquaculture, Sustainable Aquaculture Techniques*. (Sustainable Aquaculture Techniques: InTech. Available from: http://www.intechopen.com/books/sustainable-aquaculture-techniques/use-of-yeasts-as-probiotics-in-fish-aquaculture.

Naylor, R.L., Hardy, R.W., Bureau, D.P., Chiu, A., Elliott, M., Farrell, A.P., Forster, I., Gatlin, D.M., Goldburg, R.J., Hua, K. & Nichols, P.D. (2009). Feeding aquaculture in an era of finite resources. *Proceedings of the National Academy of Sciences*.

Niklasson, L., Sundh, H., Fridell, F., Taranger, G.L. & Sundell, K. (2011). Disturbance of the intestinal mucosal immune system of farmed Atlantic salmon (Salmo salar), in response to long-term hypoxic conditions. *Fish Shellfish Immunol,* 31(6), pp. 1072-80.

Nilsson, J., Brännäs, E. & Eriksson, L.O. (2010). The Swedish Arctic charr breeding programme. *Hydrobiologia,* 650(1), pp. 275-282.

Nordic Committee on Food Analysis (1976). Determination in feeds and faeces according to Kjeldahl, No6. In. Oslo, Norway: NKML.

NRC (2011). *Nutrient Requirements of Fish and Shrimp*. Washington, DC: The National Academies Press. Available from: http://www.nap.edu/catalog/13039/nutrient-requirements-of-fish-and-shrimp.

Ok, I.H., Bai, S.C., Park, G.J., Choi, S.M. & Kim, K.W. (2001). The patterns of plasma free amino acids after force-feeding in rainbow trout Oncorhynchus mykiss (Walbaum) with and without dorsal aorta cannulation. *Aquaculture Research,* 32, pp. 70-75.

Oliva-Teles, A. & Gonçalves, P. (2001). Partial replacement of fishmeal by brewers yeast (Saccaromyces cerevisae) in diets for sea bass (Dicentrarchus labrax) juveniles. *Aquaculture,* 202(3-4), pp. 269-278.

Olstorpe, M. & Passoth, V. (2011). Pichia anomala in grain biopreservation. *Antonie van Leeuwenhoek,* 99(1), pp. 57-62.

Olstorpe, M., Schnürer, J. & Passoth, V. (2009). Screening of yeast strains for phytase activity. *FEMS Yeast Research,* 9(3), pp. 478-488.

Paulsen, S.M., Lunde, H., Engstad, R.E. & Robertsen, B. (2003). In vivo effects of beta-glucan and LPS on regulation of lysozyme activity and mRNA expression in Atlantic salmon (Salmo salar L.). *Fish Shellfish Immunol,* 14(1), pp. 39-54.

Peterson, H.C., Fronc, K., Peterson, H.C. & Fronc., K. (2007). *Fishing for consumers: market-driven factors affecting the sustainability of the fish and seafood supply chain Globalization: Effects on Fisheries Resources*: Cambridge University Press.

Refstie, S., Baeverfjord, G., Seim, R.R. & Elvebø, O. (2010). Effects of dietary yeast cell wall [beta]-glucans and MOS on performance, gut health, and salmon lice resistance in Atlantic salmon (Salmo salar) fed sunflower and soybean meal. *Aquaculture,* 305(1-4), pp. 109-116.

Reverter, M., Lundh, T. & Lindberg, J.E. (1997). Determination of free amino acids in pig plasma by precolumn derivatization with 6-N-aminoquinolyl-N-hydroxysuccinimidyl carbamate and high-performance liquid chromatography. *J Chromatogr B Biomed Sci Appl,* 696(1), pp. 1-8.
Ringø, E., Olsen, R.E., Mayhew, T.M. & Myklebust, R. (2003). Electron microscopy of the intestinal microflora of fish. *Aquaculture,* 227(1–4), pp. 395-415.
Rolland, M., Feekings, J.P., Dalsgaard, J., Holm, J. & Skov, P.V. (2015). Modelling the effects of dietary methionine level and form on postprandial plasma essential amino acid profiles in rainbow trout (Oncorhynchus mykiss). *Aquaculture Nutrition*, pp. n/a-n/a.
Rumsey, G.L., Hughes, S.G. & Kinsella, J.L. (1990). Use of Dietary Yeast Saccharomyces cerevisiae Nitrogen by Lake Trout. *Journal of the World Aquaculture Society,* 21(3), pp. 205-209.
Rumsey, G.L., Hughes, S.G., Smith, R.R., Kinsella, J.E. & Shetty, K.J. (1991). Digestibility and energy values of intact, disrupted and extracts from brewer's dried yeast fed to rainbow trout (Oncorhynchus mykiss). *Animal Feed Science and Technology,* 33(3-4), pp. 185-193.
Rumsey, G.L., Winfree, R.A. & Hughes, S.G. (1992). Nutritional value of dietary nucleic acids and purine bases to rainbow trout (Oncorhynchus mykiss). *Aquaculture,* 108(1-2), pp. 97-110.
Russell, W.M.S. & Burch, R.L. (1959). The principles of humane experimental technique.
Sánchez-Muniz, F.J., de La Higuera, M. & Varela, G. (1982). Alterations of erythrocytes of the rainbow trout (Salmo gairdneri) by the use of hansenula anomala yeast as sole protein source. *Comparative Biochemistry and Physiology Part A: Physiology,* 72(4), pp. 693-696.
Satyanarayana, T., Johri, B.N. & Prakash, A. (2012). *Microorganisms in Sustainable Agriculture and Biotechnology*: Springer Netherlands.
Schuhmacher, A., Wax, C. & Gropp, J.M. (1997). Plasma amino acids in rainbow trout (Oncorhynchus mykiss) fed intact protein or a crystalline amino acid diet. *Aquaculture,* 151(1–4), pp. 15-28.
Segner, H., Sundh, H., Buchmann, K., Douxfils, J., Sundell, K.S., Mathieu, C., Ruane, N., Jutfelt, F., Toften, H. & Vaughan, L. (2012). Health of farmed fish: its relation to fish welfare and its utility as welfare indicator. *Fish Physiol Biochem,* 38(1), pp. 85-105.
Shepherd, C.J. & Jackson, A.J. (2013). Global fishmeal and fish-oil supply: inputs, outputs and marketsa. *Journal of Fish Biology,* 83(4), pp. 1046-1066.
Short, F.J., Gorton, P., Wiseman, J. & Boorman, K.N. (1996). Determination of titanium dioxide added as an inert marker in chicken digestibility studies. *Animal Feed Science and Technology,* 59(4), pp. 215-221.
Smith, L.S. & Bell, G.R. (1964). A Technique for Prolonged Blood Sampling in Free-Swimming Salmon. *Journal of the Fisheries Research Board of Canada,* 21(4), pp. 711-717.
Soivio, A., Nynolm, K. & Westman, K. (1975). A technique for repeated sampling of the blood of individual resting fish. *J Exp Biol,* 63(1), pp. 207-17.

Stadmark, J. & Conley, D.J. (2011). Mussel farming as a nutrient reduction measure in the Baltic Sea: Consideration of nutrient biogeochemical cycles. *Marine Pollution Bulletin,* 62(7), pp. 1385-1388.
Statistics Sweden (2013). *Aquaculture in Sweden 2013*: Statistics Sweden.
Stoskopf, M.K. (1993). *Fish Medicine*: W.B. Saunders Company. Available from: https://books.google.se/books?id=giMXAQAAIAAJ.
Sunde, J., Kiessling, A., Higgs, D., Opstvedt, J., Venturini, G. & Rungruangsak-Torrissen, K. (2003). Evaluation of feed protein quality by measuring plasma free amino acids in Atlantic salmon (Salmo salar L.) after dorsal aorta cannulation. *Aquaculture Nutrition,* 9(6), pp. 351-360.
Sundell, K., Jutfelt, F., Agustsson, T., Olsen, R.E., Sandblom, E., Hansen, T. & Björnsson, B.T. (2003). Intestinal transport mechanisms and plasma cortisol levels during normal and out-of-season parr-smolt transformation of Atlantic salmon, *Salmo salar*. *Aquaculture,* 222(1-4), pp. 265-285.
Sundell, K. & Sundh, H. (2012). Intestinal fluid absorption in anadromous salmonids: importance of tight junctions and aquaporins. *Frontiers in Physiology,* 3, p. 388.
Sundell, K.S. & Rønnestad, I. (2011). Intestinal absorption. In: Editor-in-Chief: Anthony, P.F. (ed. *Encyclopedia of Fish Physiology*. San Diego: Academic Press, pp. 1311-1321. Available from: http://www.sciencedirect.com/science/article/pii/B9780123745538000721 .
Sveier, Nordås, Berge & Lied (2001). Dietary inclusion of crystalline D- and L-methionine: effects on growth, feed and protein utilization, and digestibility in small and large Atlantic salmon (Salmon salar L.). *Aquaculture Nutrition,* 7(3), pp. 169-181.
Tacon, A.G. & Metian, M. (2009). Fishing for feed or fishing for food: increasing global competition for small pelagic forage fish. *AMBIO,* 38(6), pp. 294-302.
Tacon, A.G.J. & Metian, M. (2008). Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: Trends and future prospects. *Aquaculture,* 285(1-4), pp. 146-158.
Tayel, A.A., El-Tras, W.F., Moussa, S.H. & El-Agamy, M.A. (2013). Antifungal action of Pichia anomala against aflatoxigenic Aspergillus flavus and its application as a feed supplement. *Journal of the Science of Food and Agriculture,* 93(13), pp. 3259-3263.
Thorarensen, H., Gallaugher, P.E., Kiessling, A.K. & Farrell, A.P. (1993). Intestinal blood flow in swimming chinook salmon Onchorhynchus Tshawytscha and the effects of haematocrit on blood flow distribution. *Journal of Experimental Biology,* 179(1), pp. 115-129.
Torrecillas, S., Makol, A., Benitez-Santana, T., Caballero, M.J., Montero, D., Sweetman, J. & Izquierdo, M. (2011). Reduced gut bacterial translocation in European sea bass (Dicentrarchus labrax) fed mannan oligosaccharides (MOS). *Fish Shellfish Immunol,* 30(2), pp. 674-81.
Torrecillas, S., Montero, D. & Izquierdo, M. (2014). Improved health and growth of fish fed mannan oligosaccharides: potential mode of action. *Fish Shellfish Immunol,* 36(2), pp. 525-44.

Turner, J., Wood, C. & Clark, D. (1983). Lactate and proton dynamics in the rainbow trout (Salmo gairdneri). *Journal of Experimental Biology,* 104, pp. 247-268.

Uddin, S.J., Shilpi, J.A., Alam, S.M.S., Alamgir, M., Rahman, M.T. & Sarker, S.D. (2005). Antidiarrhoeal activity of the methanol extract of the barks of Xylocarpus moluccensis in castor oil- and magnesium sulphate-induced diarrhoea models in mice. *Journal of Ethnopharmacology,* 101(1–3), pp. 139-143.

Venou, B., Alexis, M.N., Fountoulaki, E. & Haralabous, J. (2006). Effects of extrusion and inclusion level of soybean meal on diet digestibility, performance and nutrient utilization of gilthead sea bream (Sparus aurata). *Aquaculture,* 261(1), pp. 343-356.

Venou, B., Alexis, M.N., Fountoulaki, E., Nengas, I., Apostolopoulou, M. & Castritsi-Cathariou, I. (2003). Effect of extrusion of wheat and corn on gilthead sea bream (Sparus aurata) growth, nutrient utilization efficiency, rates of gastric evacuation and digestive enzyme activities. *Aquaculture,* 225(1–4), pp. 207-223.

Vidakovic, A., Djordjevic, B., Seth, H., Sandblom, E., Axelsson, M. & Kiessling, A. (2011). Physiological stress response in rainbow trout to graded levels of CO2 and temperature changes. *Conference: cost annual conference*(867), p. 14.

Wilson, R.P. (2002). Amino acids and proteins. In: Hardy, J.E.H.a.R.W. (ed. *Fish Nutrition*. 3rd Edition. ed. San Diego, CA: Academic Press, pp. 143-179.

Wilson, R.P. & Cowey, C.B. (1985). Amino acid composition of whole body tissue of rainbow trout and Atlantic salmon. *Aquaculture,* 48(3–4), pp. 373-376.

Vohra, A. & Satyanarayana, T. (2001). Phytase production by the yeast, Pichia anomala. *Biotechnology Letters,* 23(7), pp. 551-554.

Yamada, S., Simpson, K.L., Tanaka, Y. & Katayama, T. (1981). Plasma Amino Acid Changes in Rainbow Trout Salmo gairdneri Force-fed Casein and a Corresponding Amino Acid Mixture. *NIPPON SUISAN GAKKAISHI,* 47(8), pp. 1035-1040.

Ytrestøyl, T., Aas, T.S. & Åsgård, T. (2015). Utilisation of feed resources in production of Atlantic salmon (Salmo salar) in Norway. *Aquaculture,* 448(0), pp. 365-374.

Øverland, M., Karlsson, A., Mydland, L.T., Romarheim, O.H. & Skrede, A. (2013). Evaluation of Candida utilis, Kluyveromyces marxianus and Saccharomyces cerevisiae yeasts as protein sources in diets for Atlantic salmon (Salmo salar). *Aquaculture,* 402-403(0), pp. 1-7.

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