Effects of feeding yeasts on blood physiology and gut microbiota of rainbow trout

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Cover: Rainbow trout with cells of yeast, blood and bacteria
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Abstract

Yeast represents a sustainable protein alternative to fishmeal in diets for farmed fish, although more than 40% replacement has been shown to reduce fish growth and welfare. This thesis investigated the effects of feeding high inclusions of inactivated and live yeasts to fish in order to replace fishmeal without negative health consequences. The specific focus was on red blood cell characteristics, plasma amino acid uptake, gut microbial communities and stress/immune responses of rainbow trout (*Oncorhynchus mykiss*).

Post-prandial blood samples were collected from dorsal aorta-cannulated rainbow trout fed diets in which 60% fishmeal protein was replaced with two yeast species, *Saccharomyces cerevisiae* and *Wickerhamomyces anomalus*, inactivated by heat extrusion. Blood analysis showed that feeding both yeasts resulted in higher blood pH and haemoglobin levels, which were associated with lower buffering capacity of yeast and possible haemolytic anaemia from metabolism of high levels of nucleic acid. Plasma analysis revealed that amino acid uptake was similar in fish fed both yeasts and fishmeal, except for higher methionine in fish fed yeasts attributed to higher supplementation. In a later study, fish were fed live *S. cerevisiae* and reared at 11 and 18°C. No adverse effects on blood physiology were found, although most cells survived digestion and were not metabolised. These results indicate that reduced growth in fish fed yeast may not be due to lower amino acid content, but rather to metabolism of high levels of nucleic acid leading to impaired red blood cell function.

In separate studies, fish were fed inactivated yeasts that replaced 20, 40 and 60% of fishmeal protein and fish kept at 11 and 18°C were fed 40% replacement with live yeast. High-throughput sequencing of the distal gut revealed that inactivated *W. anomalus* affected bacterial diversity and abundance, while both inactivated and live *S. cerevisiae* had minor effects. Increased temperature reduced the abundance of lactic acid bacteria and reduced bacterial diversity. In both studies, *Debaryomyces hansenii, S. cerevisiae* and *Rhodotorula* spp. were naturally present in the fish gut and feeding live yeast, but not inactivated, increased the gut yeast load. Fish at 18°C had higher plasma cortisol levels and suppressed expression of inflammatory cytokines, which were further suppressed when fed live yeast. This suggests that increased temperature subjected fish to chronic stress and that feeding live yeast may impair the innate immune response. In conclusion, this thesis suggest that impaired red blood cell and immune function are key factors reducing growth and welfare of rainbow trout fed yeast and managing these factors may enable sustainable replacement of fishmeal.

Keywords: rainbow trout, yeast, protein, probiotic, blood, gut microbiota, stress, temperature, aquaculture, *Saccharomyces cerevisiae, Wickerhamomyces anomalus*

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Effekter av utfodring av jäst på blodfysiologi och gutmikrobiot av regnbågeöring

Abstract

Jäst utgör ett hållbart proteinalternativ till fiskmjöl i foder till odlad fisk, men resultat presenterade i denna avhandling indikerar att inblandning med mer än 40% minskar fisktillväxten. Syftet med denna avhandling var att förstå effekten av högre halter av inaktiverad respektive levande jäst till fisk utan negativa hälsoeffekter. Specifikt effekterna på röda blodkroppar, upptag av plasmaaminosyror, tarmmikrobiota och stress/immunsvar på regnbåge (*Oncorhynchus mykiss*).

Postprandiala blodprover togs från dorsal aorta-kannulerad regnbåge som utfodrats med dieter där 60% av fiskmjölsproteinet ersatts med två olika typer av inaktiverat/avdödad jäst, *Saccharomyces cerevisiae* och *Wickerhamomyces anomalus*, gjord av värmextrudering. Blodanalys visade högre blod-pH och hemoglobinnivåer vilket associeras med jästens lägre buffertkapacitet och eventuell hemolytisk anemi på grund av ökad nukleinsyrametabolism. Plasmaanalyser visade att aminosyrornas upptag var lika mellan fisk som matades med jäst eller fiskmjöl, förutom att koncentration av plasma-metionin var högre hos fiskar som utfodrats med jäst-diet. I en senare studie utfodrades regnbåge med levande jäst (*S. cerevisiae*) vid två olika temperaturer (11 och 18°C). Inga biverkningar på blodfysiologi hittades, även om de flesta celler överlevde och inte metaboliserades i tarmkanalen. Dessa resultat indikerar att minskad fisktillväxt inte enbart kan bero på lägre aminosyrokoncentration, utan snarare en för hög nukleinsyrahalt i fodret men bara när jäst metaboliserades.

I ytterligare två studier studerades tillväxt och tarmbiota där fisk gavs foder med inaktiverad jäst som ersatte 20, 40 och 60% av fiskmjölprotein därefter utfördes försök med fisk som utfodrades vid 11 och 18°C med 40% inbladning av levande jäst i fodret. Hög genomströmnings sekvensering av distala tarmen avslöjade att inaktiverad *W. anomalus* påverkade både antal och diversitet av bakterier i tarmen medan både inaktiverad och levande *S. cerevisiae* hade mindre effekt. Ökad vattentemperatur minskade mängden mjölksyrabakterier och minskad bakteriell mångfald i tarmen. I båda studierna har *Debaryomyces hansenii, S. cerevisiae* och *Rhodotorula* spp. påträffade i fisktarmen medan fisk som utfodrats med levande jäst, bidrog till en ökad, jästbelastning i tarmen. Fisken vid 18°C hade dock högre plasmakortisolnivåer och reducerat genuttryck av proinflammatoriska cytokiner vilket var än mer påtagligt hos fisk utfodrade med levande jäst. Dessa resultat tyder på att ökad temperatur utsätter fisk för kronisk stress och att utfodring av levande jäst påverkar immunresponsen. Fisktillväxt och välfärd bibehållas genom att ge jäst i kallt vatten med lägre nukleinsyrahalt och om det lyckas kan det leda till högre ersättning av fiskmjöl.

Nyckelord: regnbågeöring, jäst, protein, probiotisk, blod, tarmmikrobiota, stress, temperatur, vattenbruk, *Saccharomyces cerevisiae, Wickerhamomyces anomalus*

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Dedication

To my parents, for encouraging my curiosity about animals with fins and feathers.

Don't stop believin'

- Steve Perry (Journey, 1981)

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 Huyben, D., Vidaković, A., Nyman, A., Langeland, M., Lundh, T.* & Kiessling, A. (2017). Effects of dietary yeast inclusion and acute stress on post-prandial whole blood profiles of dorsal aorta-cannulated rainbow trout. *Fish Physiology and Biochemistry* 43(2), 421-434.

II Huyben, D., Vidaković, A., Langeland, M., Nyman, A., Lundh, T.* & Kiessling, A. (2017). Effects of dietary yeast inclusion and acute stress on postprandial plasma free amino acid profiles of dorsal aorta-cannulated rainbow trout. *Aquaculture Nutrition* 1-11, DOI: 10.1111/anu.12551.

III Huyben, D.*, Nyman, A., Vidaković, A., Passoth, V., Moccia, R., Kiessling, A., Dicksved, J. & Lundh, T. (2017). Effects of dietary inclusion of the yeasts *Saccharomyces cerevisiae* and *Wickerhamomyces anomalus* on gut microbiota of rainbow trout. *Aquaculture* 473, 528-537.

IV Huyben, D.*, Sun, L., Moccia, R., Kiessling, A., Dicksved, J. & Lundh, T. Gut microbiota of rainbow trout is affected by high dietary inclusion of live yeast and increased water temperature. (Manuscript).

V Huyben, D.*, Vidaković, A., Sundh, H., Sundell, K., Kiessling, A. & Lundh, T. Blood and intestinal physiology of rainbow trout is affected by high dietary inclusion of live yeast and increased water temperature. (Manuscript).

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The contributions from David Huyben to the papers included in this thesis were as follows:

I Planned part of the experiment, assisted with cannulation surgeries, fed and collected waste from fish, analysed most blood parameters, performed the statistical analyses and wrote the manuscript.

II Planned part of the experiment, assisted with cannulation surgeries, fed and collected waste from fish, assisted with plasma analyses, performed the statistical analyses and wrote the manuscript.

III Planned part of the experiment, sampled fish, analysed microbiota parameters, performed the statistical analyses and wrote the manuscript.

IV Planned the experiment, carried out most feeding and collection of waste from fish, sampled gut materials, analysed microbiota parameters, performed the statistical analyses and wrote the manuscript.

V Planned the experiment, carried out most feeding and collection of waste from fish, sampled gut materials, analysed some blood parameters, performed the statistical analyses and wrote the manuscript.

Abbreviations

BE	Base excess
BW	Body weight
CFU	Colony-forming unit
DM	Dry matter
FCR	Feed conversion ratio
FI	Feed intake
Hb	Haemoglobin
Hct	Haematocrit
HSI	Hepatosomatic index
HSP	Heat shock protein
IL	Interleukin
INF	Interferon
Leu	Leucocrit
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
RBC	Red blood cell
SGR	Specific growth rate
TGC	Thermal growth coefficient
TGF	Transforming growth factor
TNF	Tumour necrosis factor
VSI	Viscerosomatic index
WG	Weight gain

1 Introduction

1.1 Aquaculture industry and feed

1.1.1 Global and Swedish aquaculture

Aquaculture, the farming of finfish, shellfish, crustaceans and aquatic plants, is one of the fastest growing food production industries worldwide, with an annual growth rate of 5.4% (FAO, 2016). In 2014, 49.8 million tonnes of finfish were farmed, representing an estimated value of $99 billion (USD) (FAO, 2016). China was the largest producer of farmed finfish in that year, supplying approximately 27.2 million tonnes or 57% of global production, followed by 2-5 million tonnes each from India, Indonesia, Vietnam, Bangladesh and Norway (FAO, 2016). The top ten most commonly farmed fish are six different carp species (family Cyprinidae), Nile tilapia (*Oreochromis niloticus*), Atlantic salmon (*Salmo salar*) milkfish (*Chanos chanos*) and rainbow trout (*Oncorhynchus mykiss*) (FAO, 2016). The majority of these fish are produced inland, in freshwater ponds and tanks, whereas Atlantic salmon and rainbow trout can be raised in both inland and coastal systems, *e.g*. marine sea cages.

In Europe, total finfish production in 2014 was only 2.3 million tonnes, or 5% of global production, with more than half supplied by Norway (FAO, 2016). In comparison, Sweden produced only 0.01 million tonnes (345 million SEK) of finfish or 0.0004% of global production (Statistics-Sweden, 2015). Swedish farmed fish consisted of 83% rainbow trout and 16% Arctic charr (*Salvelinus alpinus*), which were mainly produced inland in freshwater cages and ponds (Statistics-Sweden, 2015). Over the past decade, aquaculture production in Europe has slightly increased, whereas production has been stagnant in Sweden and even decreased by 14% from 2012 to 2015 (Statistics-

Sweden, 2015). Uncertainty over the environmental impact of aquaculture on wild populations, strong economic competition from other countries and other factors have hindered aquaculture growth in Sweden (Jordbruksverket, 2012). However, the potential for increased aquaculture in Sweden is high due to the numerous freshwater lakes, long coastline, highly educated workforce and high demand for fish. In general, aquaculture production needs to increase to meet the growing demand for fish as food, especially since fish is an important source of essential amino acids, fatty acids, vitamins and minerals for the human population. In addition, fish production in low-income regions and countries is important in order to increase economic gains and food supplies that may be lacking.

1.1.2 Feed for salmonids

In order to produce more fish to feed a growing human population, more feed resources will be needed. For many years, there has been an increased demand for fish as food for humans, fish oil supplements, livestock/pet feeds and other applications, which has led to higher prices and lower availability of fishmeal for use in fish feeds (Tacon & Metian, 2008). Feed is one of the highest costs of finfish production in intensive aquaculture systems and the protein fraction is usually the most expensive, compared with lipids and carbohydrates. In addition, using fishmeal derived from wild fish to feed farmed fish has been criticised as an unsustainable practice, since it increases pressure on the dwindling catches from fisheries and consumes a high-quality food source of human interest (Tacon *et al.*, 2011). The inclusion rate of fishmeal in fish feeds is expected to decrease due to elevated prices, increased demand and reduced availability, and thus alternative protein sources will become increasingly important to the aquaculture industry.

Reduced use of fishmeal in diets for commonly farmed omnivorous fish, such as carp and tilapia, will be less affected, since fishmeal represents a minor component (<10%) of their diet as carbohydrate sources can be relied upon (NRC, 2011; Tacon & Metian, 2008). Carnivorous fish, such as salmon and trout, will be more affected by reduced fishmeal inclusion, since it represents a major dietary component and since salmonid species require higher protein levels than omnivorous fish (NRC, 2011; Tacon & Metian, 2008). However, the use of fishmeal has been decreasing for many years, as the inclusion rate in diets for salmon and trout was approximately 40-45% in 1995 and is estimated to decrease to 12% by 2020 (Figure 1). Fishmeal, which is commonly derived from low-temperature dried herring or menhaden, contains a high level of crude protein (*i.e*. 65-75% on a dry matter [DM] basis), is highly digestible and

meets the amino acid requirements of most fish species (NRC, 2011). In addition, breeding programmes and intensive farming have improved fish growth rates and feed efficiencies, which has increased the need for highly digestible and protein-rich diets. Therefore, alternative protein sources need to be of similar quality.

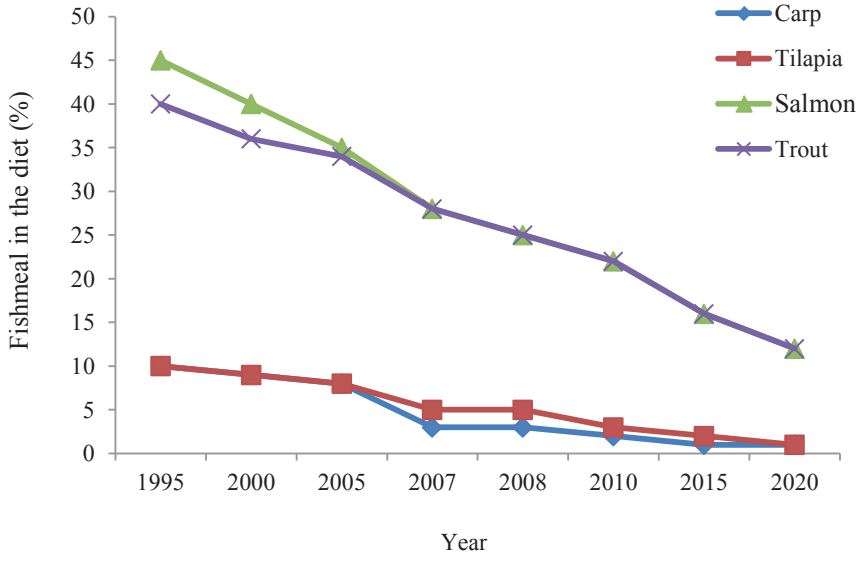


Figure 1. Estimated global use of fishmeal in the diet of carp, tilapia, salmon and trout, 1995-2020. Data from Tacon *et al.* (2011).

Plant feed sources, such as soybeans, contain moderate levels of protein and have been increasingly used to replace fishmeal in fish feeds during the past two decades (Tacon *et al.*, 2011). However, soybeans and other plants can be directly used for human consumption and increased demand has also led to increased prices and reduced availability (Tacon *et al.*, 2011). The cultivation of plants cannot expand to fulfil the demand for inclusion in fish feeds, as the planet has a limited amount of arable land that is under increasing pressure from various factors, *e.g.* climate change, pollution and soil erosion. In addition, plants contain anti-nutritional factors that can result in intestinal inflammation and reduced growth of salmonid fish (Krogdahl *et al.*, 2010). However, feed processing techniques can help to remove inhibitory compounds from plant sources, such as soy protein concentrate, but with additional costs (Hardy, 2010). Increased use of plant sources to produce biofuels and biopolymers may also lead to increased demand, higher prices and lower availability. Therefore, alternative fish feed sources of non-human

interest are needed, especially those that are widely available, environmentally sustainable and do not reduce fish health.

1.1.3 Amino acid requirement

Fish require a balanced intake of amino acids that are important for many metabolic pathways, especially protein synthesis and the supply of energy necessary for growth and immune function. Fish acquire amino acids from the diet by catabolising protein (proteolysis) into shorter peptides and free amino acids by hydrochloric acid (HCl) and various enzymes, such as peptidases and proteases, in the gastro-intestinal tract. These amino acids are absorbed by the epithelium in the small intestine and further processed in the liver (Wilson, 2002). Amino acids consist of both an amine and a carboxylic acid group with a side chain (R group) and in total there are 22 proteinogenic (protein-building) amino acids. These amino acids can be classified as essential (indispensable) if they cannot be synthesised or are inadequately synthesised by animals to required levels, or as non-essential (dispensable) if they are adequately synthesised (Li *et al.*, 2009). Essential amino acids for rainbow trout include arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. Methionine and lysine are often the most limiting amino acids in fish feeds, especially when fishmeal is replaced by plant protein sources insufficient in these two amino acids (Wilson, 2002). For this reason, methionine and lysine are usually provided in crystalline form as a supplement to meet the requirements of fish and maintain growth when fishmeal is replaced with plant protein sources.

Some non-essential amino acids can be conditionally essential and must be provided in the diet if the rate of utilisation exceeds synthesis. It has been estimated that cysteine (non-essential) can contribute 40-60% of methionine in the diet of several fish species (Wilson, 2002), and thus levels of methionine and cysteine are commonly combined. Previous research suggests that ingredients that replace fishmeal need to meet the essential and conditionally essential amino acid requirements of fish in order to be a sufficient protein alternative (reviewed by Li *et al.* (2009)). If levels of conditionally essential amino acids in the diet are not high enough, this may result in reduced fish growth. For example, dietary supplementation with hydroxy-proline (non-essential), but not proline, increases growth rate and modifies the bone composition of Atlantic salmon (Aksnes *et al.*, 2008). Serial blood sampling and analysis of plasma amino acids from fish following feeding (post-prandial) is one method to compare differences in amino acid uptake between fish fed different dietary sources.

1.2 Yeast as a protein source

1.2.1 Single cell proteins

Single cell proteins (SCP) are an inactivated, dried cell mass of microorganisms, such as micro-algae, bacteria, filamentous fungi and yeast, capable of converting low-quality organic material into a high protein food (Nalage et al., 2016). The ability of SCP to grow on organic waste presents an opportunity to recapture indispensable nutrients, such as nitrogen and phosphorus, while reducing waste and producing food for a growing human population. For centuries, live microbes have helped humans to process food, such as yeast in baking and brewing, but humans have shown little interest in consuming microbes as a food source. This low human interest suggests that SCP may be better utilised as a feedstuff for livestock. Among SCP, Bacteria have the highest content of crude protein that is similar to fishmeal and soy protein concentrate while algae, yeast and filamentous fungi have lower protein similar to soybean meal (Table 1). However, bacteria have a higher content of nucleic acids, which can negatively affect health (see Section 1.2.3). Moreover, bacteria, algae and filamentous fungi have a higher risk of contamination during cultivation than yeast and algae and filamentous fungi have lower growth rates than yeast (Nalage *et al.*, 2016; Anupama & Ravindra, 2000).

Table 1. *Nutritional composition (% dry matter basis) of microbial, animal and plant sources. Modified from Nalage et al. (2016); NRC (2011); Sauvant et al. (2002)*

Source	Crude protein	Crude lipids	Nucleic acids	Ash
Microbial				
Yeast	46-53	1-6	6-12	5-10
Filamentous fungi	31-50	2-8	7-10	9-14
Bacteria	72-78	2-3	8-16	3-7
Algae	47-63	7-20	3-8	8-10
Animal and Plant				
Fishmeal	70-78	8-10	1-2	11-21
Soy concentrate	60-69	1-3	0-1	8-9
Soybean meal	47-51	1-3	0-1	6-8
Wheat flour	13-16	1-3	2-3	1-2

Yeast is an exceptional SCP since it contains a moderate protein level, is abundant in lysine, has low toxic potential, can be cultivated on a wide range

of substrates, survives low pH, achieves high growth rates, is easy to harvest and has high consumer acceptance (Nalage *et al.*, 2016; Nasseri *et al.*, 2011; Anupama & Ravindra, 2000; Kuhad *et al.*, 1997). In addition, yeast is a source of many essential B vitamins and has a balanced amino acid profile, except for sulphur-containing amino acids. Yeast is produced on a substrate of molasses by-product, ammonia, minerals and vitamins, and can be harvested directly after fermentation or recovered after industrial applications, *e.g.* brewing and ethanol production (Nalage *et al.*, 2016; Anupama & Ravindra, 2000). *Saccharomyces cerevisiae* (baker's yeast) and *Candida utilis* (*Torula* yeast) are the most common yeasts used in animal feeds, although other yeast species have potential (Nalage *et al.*, 2016). *Phaffia rhodozyma* contains a high content of essential fatty acids and pigments required in salmonid feeds (Sanderson & Jolly, 1994; Johnson *et al.*, 1980). *Wickerhamomyces anomalus* (formerly *Pichia* and *Hansenula anomala*) is capable of inhibiting moulds during feed storage and increasing phosphorus digestibility in feeds for rainbow trout due to high activity of extracellular enzymes, especially phytase (Vidakovic *et al.*, submitted; Passoth *et al.*, 2006).

1.2.2 Yeast fed to salmonids

Yeast has high potential for use as an alternative protein source to fishmeal due to its similar amino acid profile and low demand as a human food. The use of yeast as a dietary protein for farmed fish is not a new concept, as studies have investigated this possibility since the 1970s. Species of *Candida* were the first yeasts to be used in diets for rainbow trout and they successfully replaced up to 40% of fishmeal without reductions in performance, *e.g.* growth rate and feed efficiency (Mahnken *et al.*, 1980; Matty & Smith, 1978). Since then, studies in the early 1990s have shown that *S. cerevisiae* can replace 25% and 50% of protein in diets for rainbow trout and lake trout (*Salvelinus namaycush*), respectively, without reduced fish performance (Rumsey *et al*., 1991; Rumsey *et al*., 1990). In later work in the 2010s, reduced growth and feed efficiency have been found in Atlantic salmon fed *S. cerevisiae* that replaced 40% of fishmeal protein, whereas performance was unaffected in salmon fed *C. utilis* and *Kluyveromyces marxianus* (Øverland *et al*., 2013). Recent studies investigating the use of grain distiller's dried yeast (*S. cerevisiae*) in diets for rainbow trout found that 37.5% replacement of fishmeal protein and 18% replacement of total dietary protein did not reduce fish performance (Sealey *et al*., 2015; Hauptman *et al*., 2014). For Arctic charr, it has been found that 40% replacement of fishmeal protein does not reduce fish growth or feed efficiency (Vidaković et al., 2016). For non-salmonid fishes, *S. cerevisiae* can replace up

to 50% fishmeal protein without reduced performance of Nile tilapia, common carp (*Cyprinus carpio*) and gilthead seabream (*Sparus aurata*) (Al-Hafedh & Alam, 2013; Omar *et al*., 2012; Oliva-Teles *et al*., 2006). Given the results of these previous studies using different yeast species, yeast substrates and fish species, it can be generally concluded that yeast can successfully replace 40-50% of fishmeal protein without reduced fish performance.

The reduced digestibility of yeast may be one reason for impaired fish performance when it is used to replace more than 40-50% of fishmeal. Compared with fishmeal, the digestibility coefficient of crude protein has been found to be lower for diets of *S. cerevisiae* fed to rainbow trout, Arctic charr and Atlantic salmon (Vidakovic *et al.*, submitted; Vidaković *et al.*, 2016; Øverland *et al.*, 2013). A few studies have attempted to increase digestibility by mechanically or enzymatically disrupting the yeast cell walls or by using yeast extracts without cell walls. A study on lake trout found that mechanical disruption of yeast improved growth and feed efficiency (Rumsey *et al.*, 1990). However, a study on Arctic charr found that feeding pure yeast extract, without cell walls, increased digestibility but at the expense of reduced fish growth (Vidaković *et al*., 2016). In contrast, a study on Nile tilapia fed yeast extract that replaced 100% of fishmeal protein and supplemented amino acids found that this did not reduce fish performance (Trosvik *et al*., 2012).

For many years, the lack of sulphur-containing amino acids, specifically methionine, in yeast has been suggested to inadequately meet the requirements to sustain fish growth, and thus crystalline amino acids are commonly added to the diet (Øverland & Skrede, 2016). A few studies have shown increased fish performance when yeast diets are supplemented with crystalline methionine compared with diets without supplementation (Murray & Marchant, 1986; Spinelli *et al.*, 1978). However, other studies have shown no effect on fish performance for diets supplemented with methionine (Vidakovic *et al.*, submitted; Oliva-Teles *et al.*, 2006; Mahnken *et al.*, 1980). Even in studies where digestible protein and methionine are balanced between fishmeal and yeast diets, rainbow trout fed higher than 40% replacement with yeast have been shown to have reduced performance (Vidakovic *et al.*, submitted; Hauptman *et al.*, 2014). These studies suggest that lower methionine content in yeast may not be a problem as long as total content in the diet is sufficient. Diet formulations with a mixture of animal and plant ingredients, especially fishmeal and wheat gluten, contribute high levels (*i.e*. 2-3% of protein) of methionine that may meet the amino acid requirement of rainbow trout, *i.e*. 0.7% dry matter basis (NRC, 2011), and thus supplementation may not be required. Therefore, deficiencies in other nutrients or the presence of anti-nutritional factors in yeast may be the cause of reduced fish performance.

1.2.3 Negative effects of yeast

One of the main concerns about using SCP at high dietary inclusion rates is the high content of nucleic acids. Nucleic acids, such as DNA and RNA, are essential for life and are composed of chains of nucleotide monomers that contain a five-carbon sugar, a nitrogenous base (pyrimidines and purines) and a phosphate group. The SCP can contain up to 16% (dry matter basis) of nucleic acid, mainly RNA, and bacteria and yeast contain the highest content of nucleic acids (Table 1) (Nalage *et al.*, 2016; Kuhad *et al.*, 1997).

In humans, a high content of dietary nucleic acid results in elevated plasma uric acid (hyperuricaemia) and formation of crystals of urate in joints and tissues, causing gout arthritis and kidney stones (Fox, 1981; Clifford & Story, 1976; Waslien *et al.*, 1970). In theory, fish should be able to metabolise high concentrations of nucleic acid safely, due to higher uricase activity in the liver than other animals (Rumsey *et al.*, 1991; Kinsella *et al.*, 1985). However, studies have found that feeding yeast to replace 100% of protein in the diet of rainbow trout results in harmful levels of uric acid in the kidney and haemolytic anaemia in the blood (Sanchez-Muniz *et al.*, 1982; De la Higuera *et al.*, 1981). Increased catabolism of purines and production of reactive oxygen species, such as hydrogen peroxide, are suggested to damage red blood cells due to insufficient reduction by antioxidant agents, *e.g.* peroxidase (Sanchez-Muniz *et al.*, 1982). High concentrations of exogenous nucleic acids and free adenine, a purine derivative, have been found to reduce feed intake and growth of rainbow trout (Rumsey *et al.*, 1992; Tacon & Cooke, 1980). In contrast, small amounts of nucleic acid can be beneficial to fish as a source of nitrogen and non-essential amino acids (Rumsey *et al.*, 1992) and exogenous supplementation of nucleotides has been shown to improve immune response and disease resistance (reviewed by Li and Gatlin (2006)). However, the high level of nucleic acids in yeast may limit its use as a protein source at high inclusion rates in salmonid diets, although other factors may also be at play.

Aside from reduced digestibility and high nucleic acid content, reduced pellet quality, and consequently reduced palatability and feed intake, may limit the use of yeast as a protein source. Two studies that fed yeast to sunshine bass and rainbow trout to replace 100% and 50-75% of protein found decreased feed intake and observed instances where fish ingested and then regurgitated the yeast diets (Gause & Trushenski, 2011; Rumsey *et al.*, 1991). Other studies have found that pellet quality decreases as yeast inclusion rate in the diet increases, resulting in reduced lipid absorption and increased pellet losses (Vidakovic *et al.*, submitted; Hauptman *et al.*, 2014). Compared with cold pelleting, feed extrusion applies high heat and pressure that has been suggested to disrupt yeast cells and increase digestibility. However, studies have found

that increased extrusion temperature has little effect on yeast cell wall disruption and utilisation of dietary astaxanthin pigment, and that yeast needs to be enzymatically disrupted before extrusion (Storebakken *et al.*, 2004a; Storebakken *et al.*, 2004b). Research is still lacking on the optimal extrusion conditions required to produce palatable fish diets with high inclusion rates of yeast.

1.3 Gut microbiota analyses and composition

1.3.1 Microbial quantification and identification methods

In the past, identification of bacteria and yeasts were difficult and labour-intensive, since the process was based on morphological and physiological properties of isolated colonies (Zhou *et al.*, 2014). For culture-dependent methods, the quantity and relative identity of microbial groups can be determined by serially diluting samples and incubating them on general or selective nutrient agar or broth cultures for a specific time and temperature, followed by counts of colony-forming units (CFU). These counts or most-probable number provide the live or culturable microbial load in the original sample after adjusting for dilution. The identity of microbes can be further determined by identifying physical characteristics, such as colour and shape, by eye or under the microscope and/or by identifying biochemical characteristics, such as the ability to metabolise different sugars. Today, these culture-dependent methods are still being used, at least to isolate certain microbes, since they are inexpensive, require few resources and the results are quantitative (Zhou *et al*., 2014). However, culture-dependent methods require a large number of isolates to provide meaningful data, not all microbes are culturable and metabolic plasticity of microbes may introduce error (Zhou *et al*., 2014). Studies have found that less than 1% of bacteria from saltwater, freshwater and soil environments are culturable (reviewed by Amann *et al.* (1995)), thus there is an inherent bias in culture-based methods. Direct microscopic counts of stained or fluorescence-labelled microbes using a gridded chamber have been suggested to give more realistic counts than culture-based methods, but microscopic counts are also labour-intensive (Amann *et al.*, 1995). In addition, microscopically visualised cells may be viable, but do not form visible colonies on plate cultures (non-culturable).

Identification of microbes using molecular-based methods has been increasing in the past few decades and a variety of techniques are available. Cultured-based methods can be combined with molecular methods to identify

cultured isolates, such as polymerase chain reaction (PCR) with chain termination (Sanger) sequencing, but do not identify microbial communities. The PCR method involves DNA extraction and cyclic amplification of specific rRNA genes using polymerases, nucleotides and targeted primers. Sequencing targets specific regions to identify microbial taxonomy, such the V4/V5 region of 16S rRNA genes that form part of the 30S small subunit (prokaryotes) and the D1/D2 region of 26S rRNA genes that form part of the nuclear large subunit (eukaryotes) (Navarrete & Tovar-Ramírez, 2014). Other regions and subunits are commonly sequenced too, *e.g*. 23S and 18S rRNA genes. However, both DNA extraction and PCR processes include their own biases since microbes do not lyse equally and primer binding can be selective, and these discriminations are amplified with each PCR cycle (Amann *et al.*, 1995). In addition, Sanger sequencing cannot identify large sample sizes and produces only a few hundred sequences per sample, which makes it difficult to compare microbial communities (Zhou *et al.*, 2014).

To identify microbial isolates without PCR, matrix-assisted laser desorption/ionisation with time-of-flight mass spectrometry (MALDI-TOF MS) can be used (reviewed by Wieser *et al.* (2012)). Using lasers, the MALDI-TOF MS method identifies molecules based on their fragmentation and the time required for them to reach the detector. However, MALDI-TOF MS is dependent on culturable microbes and it can become expensive to identify large sample sizes.

To determine numerical data, quantitative real-time PCR (qPCR) can be performed by synthesising complementary DNA from mRNA using reverse transcriptase followed by PCR with primers and fluorescent dyes or probes that target specific genes. The qPCR approach can provide quantitative data on microbial load in place of culture-based methods and qPCR can also be used to determine expression levels of other cells, *e.g*. cytokines involved in immune response. However, only known gene sequences can be targeted with qPCR and expression can vary between tissues (Zhou *et al.*, 2014).

In the past, denaturing- and temperature-gradient gel electrophoresis (DGGE/TGGE) was commonly used to identify microbial communities. This method is based on separation of PCR products in a gel depending on their base pair sequence. Similarly, restriction fragment length polymorphism (RFLP) uses electrophoresis, but in a preliminary step enzymes are used to cut sequences at specific sites. However, DGGE/TGGE and RFLP are becoming obsolete due to the emergence of inexpensive DNA sequencing technologies.

Recently, increases in the affordability and efficiency of high-throughput sequencing methods have increased their application in mapping microbial communities in several animals and environments (reviewed by Metzker

(2010)). Pyrosequencing (454) was initially developed, but is currently being replaced by more efficient methods, *e.g*. sequencing-by-synthesis (Illumina and Ion Torrent platforms). These methods can be used to sequence entire rRNA genes in order to determine the function of microbial communities in a metagenomics approach or the same region on rRNA genes can be sequenced for thousands of microbes to identity communities of microbes in a meta-barcoding approach (Caporaso *et al.*, 2011). For the meta-barcoding approach, the Illumina platform involves individual tagging of samples with a unique barcode during PCR amplification, which allows hundreds and even thousands of samples to be pooled and sequenced. Illumina sequencing typically results in tens of thousands of sequences per sample and returns in the order of 100 million sequencing reads per flow cell (Caporaso *et al.*, 2011). A bioinformatics pipeline needs to be in place to de-multiplex and quality filter the data, which requires a high degree of computing knowledge, power and storage. These data provide quantitative and taxonomic information down to the genus or species level, depending on the platform and primer set, which is important in determining changes in α-diversity (*e.g*. number of taxa) and β-diversity (*e.g*. species composition) of microbial communities. Drawbacks of this method are that it is time-consuming, includes DNA extraction and PCR biases, requires expensive sequencers and involves complex data handling (Zhou *et al.*, 2014; Metzker, 2010).

1.3.2 Gut microbiota in salmonids

Animals harbour many different microbes in their gastrointestinal tract, such as bacteria, yeast, viruses, protozoans and archaeans, and the loads are especially high in the intestine (hereafter referred to as the 'gut'). These microbes influence various host functions, including gut development, feed digestion, nutrient supply, immunity and disease resistance (Romero *et al.*, 2014; Berg, 1996). The microbial community in the gut can be classified into two groups: those that pass though the gut as transient content (allochthonous microbiota) and those that reside and associate with host tissues (autochthonous microbiota) (Berg, 1996). Microbes that persist in the gut of most individuals of a population or species and do not cause harm to the host are referred to as 'normal' gut microbiota (Berg, 1996).

Several studies have found that microbial composition in the fish gut is influenced by many factors, *e.g*. rearing system, fish species, life stage and diet (reviewed by Romero *et al.* (2014) and Nayak (2010)). In addition, microbial composition can be influenced by the gut region, *i.e*. proximal (anterior or midgut) or distal (posterior or hindgut) (Gajardo *et al.*, 2016; Andlid *et al.*,

1995). Microbial composition can also depend on the type of gut material, *e.g.* content, faeces, mucosa and mucus. For example, the bacterial load in the gut content of rainbow trout has been reported vary between 6 and 8 log CFU g^{-1}, respectively, based on DGGE methods (Merrifield *et al.*, 2009; Huber *et al.*, 2004) and illustrates large variations between fish within the same populations. Yeast species are often reported in studies seeking to identify fish gut microbiota, but their presence is variable as yeast load can range from below the detection limit to up to 7 log CFU g^{-1} (Gatesoupe, 2007). Yeast species may account for approximately 1% of total microbial isolates, but yeast cells are over 100-fold larger than bacterial cells, *e.g.* 200-300μm^3 for *S. cerevisiae* compared with 1μm^3 for *Pseudomonas* (Gatesoupe, 2007). Thus, the contribution of yeast to the gut microbiota is often underestimated.

Before 2013, studies that examined the gut bacteria of salmonids used electrophoresis-based methods, such as DGGE, TGGE and RFLP, and found that bacteria were commonly represented by the phyla Proteobacteria and Firmicutes, while Actinobacteria, Bacteroidetes, Fusobacteria and Tenericutes were less frequently reported (Figure 2) (Nayak, 2010). In these studies, gut bacteria commonly reported include the genera *Acinetobacter*, *Aeromonas*, *Bacillus*, *Carnobacterium*, *Citrobacter*, *Clostridium*, *Delftia*, *Lactobacillus*, *Lactococcus*, *Micrococcus*, *Pseudomonas*, *Ralstonia*, *Shewanella*, *Sphingomonas* and *Staphylococcus* (Navarrete *et al.*, 2012; Navarrete *et al.*, 2010; Merrifield *et al.*, 2009; Heikkinen *et al.*, 2006; Pond *et al.*, 2006; Huber *et al.*, 2004). Lactic acid bacteria (order Lactobacillales), such as *Lactobacillus* and *Lactococcus*, make up a significant proportion of fish gut bacteria and this group has been shown to be beneficial in the gut as they produce bacteriocins that are antagonistic toward pathogens (Ringø & Gatesoupe, 1998).

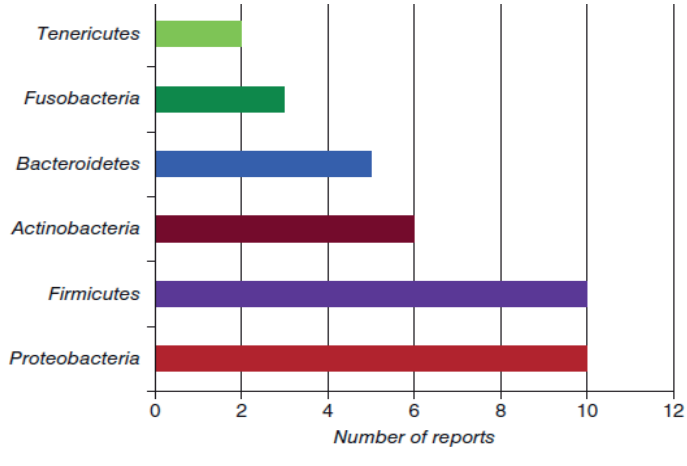


Figure 2. Bacterial phyla reported in the gut of salmonid fishes. Modified from Nayak (2010).

To date, yeast species present in the gut of salmonids have only been identified using Sanger sequencing and biochemical-based methods and those found are mainly represented by the phyla Ascomycota and Basidiomycota (Gatesoupe, 2007). In rainbow trout, common genera of *Saccharomyces, Rhodotorula, Cryptococcus*, *Debaryomyces* and *Leucosporidium* have been found in the gut (Aubin *et al.*, 2005; Andlid *et al.*, 1995). *Debaryomyces hansenii* is one of the most commonly isolated yeast species in the gut of salmonids, followed by *S. cerevisiae* and *Rhodotorula* spp. (Gatesoupe, 2007).

Within the past four years, high-throughput sequencing has been applied to map bacterial communities in the gut of rainbow trout. As in previous studies, bacteria identified as Proteobacteria and Firmicutes have been found in high abundance, except when Tenericutes is present (Figure 3) (Lyons *et al.*, 2017; Michl *et al.*, 2017; Lyons *et al.*, 2016; Lowrey *et al.*, 2015; Lyons *et al.*, 2015; Ingerslev *et al.*, 2014; Wong *et al.*, 2013). Genera found in the gut using high-throughput sequencing are also similar to those reported in previous studies, except that more lactic acid bacteria, specifically *Leuconostoc* and *Streptococcus*, and *Photobacterium* have been found. However, different fish rearing, gut sampling, DNA extraction, PCR and sequencing techniques make it difficult to compare studies. Studies that use both electrophoresis and sequencing-based methods have found *Mycoplasma*, part of the Tenericutes phylum, which seems to be either dominant (*i.e.* 78-91%) or absent in the gut of rainbow trout.

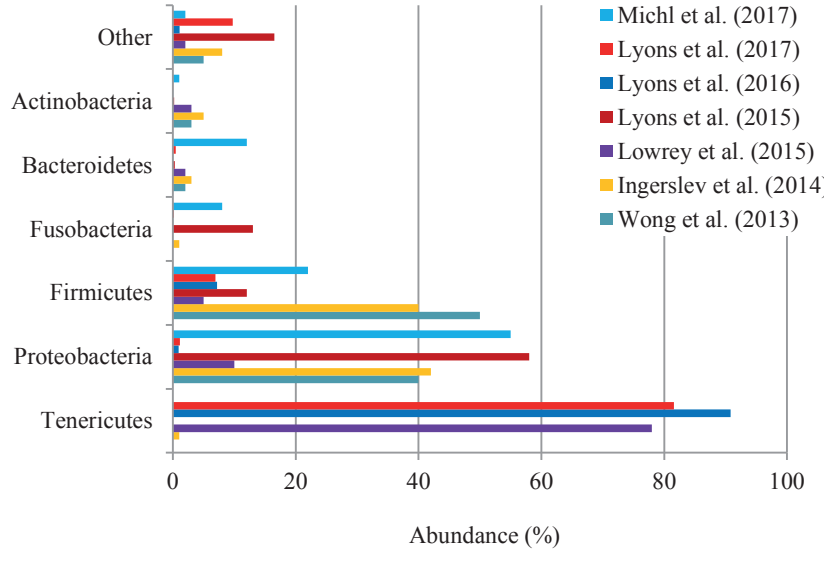


Figure 3. Mean relative abundance of bacterial phyla reported in the gut of rainbow trout using high-throughput sequencing. Data compiled from the sources listed in the legend.

1.3.3 Diet and temperature effects on gut microbiota

A few studies have investigated the effect of diet, either using alternative proteins or probiotics, on the gut microbiota of rainbow trout using high-throughput sequencing. Diets that replaced 10% of fishmeal with pea meal resulted in higher abundance of Firmicutes, specifically *Streptococcus*, *Leuconostoc* and *Weissella*, while bacterial diversity remained unchanged (Ingerslev *et al.*, 2014). In that study, the probiotic *Pediococcus acidilactici* was fed with and without pea meal and no differences in bacterial abundance and diversity were found (Ingerslev *et al.*, 2014). Diets that replaced 50 and 97% of fishmeal with plant proteins (pea, rapeseed and wheat gluten) resulted in higher abundance of order Lactobacillales, Bacillales and Pseudomonadales and decreased bacterial diversity (Michl *et al.*, 2017). In contrast, diets that replaced 1% of plant meal with intact micro-algae, a SCP, resulted in higher abundance of *Streptococcus*, *Leuconostoc*, *Lactobacillus*, *Lactococcus* and *Weissella* and higher bacterial diversity (Lyons *et al.*, 2016). Based on these studies, high inclusion of plant protein appears to alter gut microbiota and reduce diversity, whereas SCP may be beneficial, but more research is needed.

Water temperature has been shown to affect the load and abundance of gut microbes in fish, but only in certain cases. Two studies have shown lower load of lactic acid bacteria in the gut of Atlantic salmon when the water temperature was increased from 9-12°C to 18-19°C, while total bacterial load remained the same (Neuman *et al*., 2016; Hovda *et al*., 2012). In addition, the total bacterial and lactic acid bacterial load remained unchanged in three species of carp and in channel catfish (*Ictalurus punctatus*) when the water temperature was increased from 4-10°C to 23-28°C, although abundance of specific lactic acid bacteria changed (Hagi *et al*., 2004). Increased water temperature seems to have an impact on lactic acid bacteria in the gut of Atlantic salmon, but research is lacking regarding rainbow trout.

1.4 Fish stress and immune function

1.4.1 Stressors and stress indicators

Stress response in fish can be expressed as changes in behaviour, such as swimming patterns, but this can be difficult to detect. Therefore, physiological and biochemical analyses, such as fish growth and blood characteristics, are commonly evaluated. The stress response in fish is mediated by the hypothalamic-pituitary-interrenal axis, where a stressor triggers the release of catecholamines (adrenaline and noradrenaline) from the chromaffin tissue and

is accompanied by the release of adrenocorticotrophic hormone from the pituitary gland and subsequent synthesis and secretion of cortisol, a corticosteroid, from the interrenal tissue (Iwama *et al.*, 2011; Perry & Bernier, 1999). Plasma adrenaline and cortisol are primary indicators of stress and are commonly analysed in studies with fish, since initiation of the stress response causes immediate changes in these parameters (Barton & Iwama, 1991). Secondary indicators include metabolic (*e.g.* plasma glucose), haematological (*e.g.* haematocrit (Hct), haemoglobin (Hb), red blood cell (RBC) counts), hydro-mineral (*e.g.* potassium) and structural (*e.g.* relative body indices) types, while tertiary indicators are apparent over a longer period of time and include fish growth and metabolic rate (Barton & Iwama, 1991).

Farmed fish are exposed to a number of stressors during their production cycle that may elicit different responses, either acute or chronic, that may or may not impair their health and welfare. In general, stress is defined as a reaction by fish to a stimulus and this response alters the homeostatic state of the fish (Barton & Iwama, 1991). Examples of common external stimuli that induce a stress response in fish include: handling or chasing fish with a net, too low or too high stocking density, poor water quality, long-term feed deprivation and water temperatures outside their preferred range (Huntingford *et al.*, 2006). Acute stress is defined as an exposure to a brief stimulus followed by short-term recovery, whereas fish subjected to chronic stress cannot escape or adapt to the stressful stimulus over a long-term period and this results in reduced fish health and welfare, *e.g.* appetite loss, impaired growth and immune suppression (Huntingford *et al.*, 2006).

Numerous improvements in fish welfare and the widespread application of welfare indicators on fish farms in recent decades have resulted in increased fish production and health. Frequent stressors, such as handling and crowding, can be reduced to improve fish welfare, but uncontrollable effects, such as global warming and ocean acidification due to consequences of climate change, will be increasingly difficult to mitigate. Most fish farms are outdoors and vulnerable to climate effects. Increased water temperature may disproportionately affect farms of salmonid fish, since they are more adapted to colder waters than carp and tilapia species (Jobling, 1981). Increased water temperature above the preferred temperature range can stress fish, while prolonged exposure can result in reduced growth and increased risk of disease (Jobling, 1981).

Diet can also act as a stressor if it is deficient in essential nutrients and/or contains compounds that negatively affect fish health. Vitamin and mineral deficiencies can make fish vulnerable to skeletal disorders and immune suppression, which increases the risk of disease and mortality (NRC, 2011).

Studies have suggested that nutrient deficiency may be enhanced when fish are exposed to stressors that bring about a greater reliance on conditionally essential amino acids involved in the immune response (Li *et al.*, 2009). As mentioned previously, anti-nutritional factors in plant sources, such as soybean meal, can cause gut enteritis that reduces nutrient absorption and fish growth (Krogdahl *et al.*, 2010). In contrast, some dietary components can enhance the immune response and improve disease resistance, *e.g.* yeast derived β-glucans (reviewed by Ringø *et al.* (2011)). In addition to physical and chemical stressors, there is an important interaction between diet and stress that may have positive or negative effects on farmed fish.

1.4.2 Gut-microbe interactions and the immune response

The fish gut is important for osmoregulation and nutrient uptake and serves as a primary barrier against the environment. The intestinal barrier is composed of three main parts; the mucus layer and the residual (autochthonous) microbiota, the physical barrier consisting of enterocytes and the underlying gut-associated lymphoid tissue (GALT) (Nayak, 2010). Enterocytes line the surface of the gut with microvilli that absorb water and nutrients and these cells secrete mucus that contains lysozymes, antimicrobial peptides and immunoglobulin M, which inhibit pathogens (Uribe *et al.*, 2011). In fish, GALT consists principally of lymphocytes, eosinophil granular cells, several types of granulocytes and plasma cells involved in innate and acquired immunity (Zapata *et al.*, 2006). Gut microbes play an important role in the development and maturation of GALT, which mediates a variety of host immune functions (Rhee *et al.*, 2004). For example, dietary supplementation with beneficial microbes (probiotics) at early developmental stages in fish has been shown to increase the numbers of gut T-lymphocytes and granulocytes and modulate immune-related genes (Picchietti *et al.*, 2009; Picchietti *et al.*, 2007).

In salmonids, the innate immune response is initiated by pro-inflammatory cytokines, such as tumour necrosis factor-α (TNFα), interferon-γ (IFNγ) and interleukin-1β (IL1β), which trigger the inflammatory process via T-lymphocyte pathways (reviewed by Uribe *et al.* (2011)). These cytokines attract other innate immune cells, such as neutrophils and macrophages, capable of secreting antimicrobial substances and phagocytising microbes. In rainbow trout, injections of IL1β have been shown to activate the hypothalamic-pituitary-interrenal axis and induce the release of cortisol, which is involved in the stress response, as previously mentioned (Holland *et al.*, 2002). Anti-inflammatory cytokines, such as transforming growth factor-β (TGFβ) and IL10, are induced in order to reduce the innate immune response

and prevent an over-reaction (Bogdan *et al*., 1992). In rainbow trout, both TGFβ and IL10 can be induced in fish immersed in water containing plasmid DNA, lactoferrin and β-glucans (Zhang *et al*., 2009). In addition, heat shock proteins (HSP) have been shown to increase in response to stress in order to aid in cell signalling and repair (Lund & Tufts, 2003).

Probiotics, live microbes that benefit gut microbiota and the host, and immunostimulants, such as β-glucans derived from yeast cell walls, have been fed to farmed fish in recent years to improve gut health and the immune response. Low inclusions of live *S. cerevisiae* yeast rather than inactivated yeast have been found to reduce intestinal inflammation in Nile tilapia, based on increased microvilli length and density as well as decreased relative expression of pro- and anti-inflammatory cytokines (TNFα and TGFβ) and HSP70 (Ran *et al*., 2015). It has also been found that feeding a low inclusion of live *C. utilis* yeast to Atlantic salmon can counteract the symptoms of soybean meal-induced enteritis (SBMIE), such as reduced microvilli oedema and atrophy, as well as maintaining the expression of amino acid, fat and drug metabolism pathways (Grammes *et al.*, 2013). Feeding yeast-derived β-glucans to common carp has been reported to result in increased expression of TNFα and IL1β in the head kidney and decreased expression in the gut after pathogen challenge (Falco *et al.*, 2012). These studies indicate that low dietary inclusions of live yeast and yeast-derived β-glucans are beneficial to fish, although studies that have fed high inclusions of inactivated yeast (40 and 60% replacement of fishmeal) to Arctic charr and rainbow trout have reported impaired gut barrier function and oedema of microvilli (Vidakovic *et al.*, submitted; Vidaković *et al.*, 2016). However, these studies only fed salmonids inactivated yeast, while effects of live yeast at high dietary inclusions on gut health and immunity of rainbow trout are unknown.

1.5 Aims of the thesis

The overall aim of this thesis was to determine the effects of feeding high inclusions of inactivated and live yeast on the blood physiology and gut microbiota of rainbow trout. A series of experiments were conducted in an attempt to identify possible mechanisms that reduce growth and welfare of rainbow trout fed diets that replace 40% or more of fishmeal protein with yeast. In Papers I-V, specific objectives were to:

- Determine the effects of feeding inactivated and live yeast on blood pH, electrolytes and haematological parameters (Papers I & V).
- Investigate whether feeding inactivated yeast results in different post-prandial profiles of plasma amino acids (Paper II).
- Test whether high dietary levels of yeast affect the acute stress response (Papers I and II) or chronic stress response (Paper V).
- Define the yeast composition in fishmeal and yeast diets produced by heat extrusion and cold pelleting (Papers III and IV).
- Determine whether graded levels of inactivated yeast, different yeast species and live yeast alter gut microbiota (Paper III and IV).
- Investigate the effects of feeding live yeast on the innate immune response and interactions with increased water temperature (Paper V).

2 Materials and methods

2.1 Experimental design

Table 2. *Information on the fish, diets, facilities, sampling regimes and analyses used in Papers I-V of this thesis*

	Papers I & II	Paper III	Papers IV & V
Fish species	Rainbow trout	Rainbow trout	Rainbow trout
Initial body weight	849g	94g	129g
Period	4 weeks	10 weeks	6 weeks
Water temperature	15°C	13°C	11 & 18°C
Number of diets	3	7	2
Replicates, total tanks	5, 15	3, 21	4, 16
Yeast species	*S. cerevisiae* & *W. anomalus*	*S. cerevisiae* & *W. anomalus*	*S. cerevisiae*
Fishmeal replacement	60%	20, 40 & 60%	40%
Feed production	Heat-extrusion	Heat-extrusion	Cold-pelleting
Material sampled	Arterial blood	Diets & distal gut	Venous blood, proximal & distal gut materials
Analyses	Blood gases, electrolytes, RBC indices & plasma amino acids	Diet & gut microbiota	Fish growth, RBC indices, gut microbiota & gene expression

The experiments reported in Papers I-V were all performed with rainbow trout reared in freshwater systems, fed diets based on fishmeal or yeast, and either blood and/or gut microbiota were collected and analysed. Papers I and II are based on the same study, as are Papers IV and V (see Table 2 for more information).

2.2 Fish and facilities

The experiments in Papers I, II, IV and V were carried out at the Aquatic Facility in the Veterinary Medicine and Animal Science Centre of the Swedish University of Agricultural Sciences (SLU) in Uppsala, Sweden. Fish were acquired from a commercial fish farm, Vilstena fiskodling AB (Fjärdhundra, Sweden), and then raised in 500-L square holding tanks. Groups of fish were distributed to 200-L oval, experimental tanks that were equipped with LED light and partial shade and received a water flow of 5-10L min^{-1} (flow-through system). The freshwater was taken from municipal groundwater and analysed for temperature, dissolved oxygen and pH using hand-held probes (Hach Lang AB, Sköndal, Sweden). Water temperature was adjusted to 15°C in Papers I-II, while in Papers IV-V it was set to 11 or 18°C. The water temperature fluctuation throughout the experiments was <1°C. The pH was consistent at approximately 8.1 in all studies, but the differences in water temperature changed the dissolved oxygen content from 10.4mg L^{-1} in Papers I-II to 9.7 or 8.6mg L^{-1} in Papers IV-V. In Papers I-II, fish were acclimatised to the experimental tanks for several months with step-wise removal of fish until one remained that would be fitted with a cannula (849g mean weight) for the four-week study, whereas in Papers IV-V 15 fish (129g) were acclimatised for three weeks while the water temperature was adjusted.

The experiment described in Paper III was carried out at the Kälarne Aquaculture Research Station (Vattenbrukscentrum Norr AB, Kälarne, Sweden), where fish were hatched and raised in a flow-through system. A total of 35 fish (94g) were allocated to 340-L square experimental tanks and acclimatised for three weeks. Water temperature was approximately 13°C, but varied between 10 and 14°C since the water was taken from a river derived from a nearby lake.

2.3 Diets and feeding

In all five papers, there was a control diet composed of 30% fishmeal. Yeast, either *S. cerevisiae* or a 70:30 mixture of *W. anomalus* and *S. cerevisiae*, was produced by Jästbolaget AB (Sollentuna, Sweden) by fermentation on

molasses, ammonia, phosphorus, magnesium and vitamins and then dried with a fluidised bed dryer. The yeast replaced fishmeal at rates of 20, 40 and 60% on a digestible protein basis of 380g kg^{-1} (DM), based on a 95 and 86% apparent digestibility coefficient of salmonids for fishmeal and yeast ingredients, respectively (Langeland *et al.*, 2016; Vidaković *et al.*, 2016; NRC, 2011). Starch and α-cellulose ingredients were varied in the yeast diets to obtain iso-nitrogenous diets. A 2:1 ratio of fish oil and rapeseed oil were used as lipid sources and the inclusion rate of fish oil was increased for the yeast diets in order to obtain iso-energetic diets (see Table 3 for diet formulation and proximate analysis).

Diets used in Papers I, II and III were produced at the Natural Resources Institute Finland (Laukaa, Finland) using a twin-screw extruder (3 mm pellets) that applied a temperature of 120-130°C to the wet mash. Diets were lipid-coated and air-dried at 60°C overnight. Samples of ingredients and extruded diets were collected for microbial analysis.

The diets used in Papers IV and V produced at the Feed Science Laboratory at SLU (Uppsala, Sweden) using a meat grinder (3mm pellets). Gelatin was used as binding agent, dissolved in hot water that increased the temperature of the wet mash up to 65°C. Diets were air-dried at 50°C for 12 hours and then chopped and sieved. Samples were collected for microbial analysis. Diets were analysed for dry matter, crude protein, crude lipid, neutral detergent fibre, ash and gross energy using methods applied by the Department of Animal Nutrition and Management at SLU (see Papers I-V for more detailed information).

Diets were distributed to fish in Papers I and II by automatic belt feeders (Hølland Teknologi AS, Sandnes, Norway) at 1.5% of body weight (BW) per day over 1.5 hours for four weeks. In Paper III, diets were distributed by automatic rotating-drum feeders at 1.5% of BW per day over 12 hours for 10 weeks and rations were increased daily based on estimated thermal growth coefficients (TGC) according to Cho (1992). In Papers IV and V, diets were distributed by automatic belt feeders at 1.5% of BW per day over 3 hours for six weeks and rations were increased weekly based on estimated TGC. In each paper, automatic belt collectors (Hølland Teknologi AS, Sandnes, Norway) were used to collect feed waste in order to calculate feed intake. Feed losses were calculated based on the feed recovery method according to Helland *et al.* (1996).

Fish were weighed before and after the experiments in all cases, except in Paper III the fish were weighed an additional two times in the middle of the experiment (see Section 2.5 for fish growth performance calculations).

Table 3. *Diet formulation and proximate analysis of the three diets used in Papers I and II. The diets were based on fishmeal (FM), 60% fishmeal replacement with* Saccharomyces cerevisiae *yeast (SC) or 60% fishmeal replacement with* Wickerhamomyces anomalus *with* S. cerevisiae *yeast (WA). See Paper III for 20 and 40% diets and Papers IV and V for 40% live SC diet*

	Diet		
Ingredients (g kg^{-1} as-is basis)	FM	SC	WA
Fishmeal	300	120	120
S. cerevisiae yeast	-	321	-
W. anomalus & S. cerevisiae yeast	-	-	355
Soy protein concentrate	135	135	135
Wheat gluten	120	120	120
Wheat starch	100	10	0
Wheat meal	60	60	60
Fish oil	110	125	124
Rapeseed oil	50	50	50
Titanium oxide	5	5	5
Mineral-vitamin premix	15	15	15
Monocalcium phosphate	10	10	10
α-Cellulose	93	24	0
L-methionine	2	5	6
Proximate composition (g kg^{-1} DM basis)			
Dry matter	924	913	933
Crude protein	425	454	463
Crude lipid	196	203	186
Neutral detergent fibre	114	45	25
Ash	68	63	62
Gross energy (MJ kg^{-1})	24	24	24

2.4 Fish sampling and analyses

2.4.1 Blood and plasma

Fish in Papers I and II had surgery before the experiments to install a cannula in their dorsal aorta for repetitive and undisturbed blood sampling (Figures 4 and 5). The cannulation procedure was based on the method by Soivio *et al.* (1975) with modifications by Djordjevic *et al.* (2012); Kiessling *et al.* (2003); Kiessling *et al.* (1995). Step-by-step, each fish was sedated with metomidate and tricaine methane sulphonate (MS222) and then transferred to a surgery bath with recirculating cold water and MS222. Local injections of lidocaine

were given in the roof of the mouth and a 1-m polyethylene tube was inserted into the dorsal aorta using a guide wire. The cannula was looped through a puncture hole and tube in the snout of the fish. Heparinised saline was injected into the cannula and sealed and the fish was returned to the tank. Blood was collected from the cannula without disturbing the fish. The cannula was cut, heparin was removed and 0.35mL of blood was collected at 0, 3, 6, 12 and 24 hours after feeding. The cannula was then again injected with heparinised saline, sealed and placed back into the fish tank. Over three weeks, fish were fed each diet for seven days and then blood was collected on day 7. For an additional week, fish were fed the same diet as the previous week and then netted for 1 minute outside the tank to induce an acute stress response after feeding.

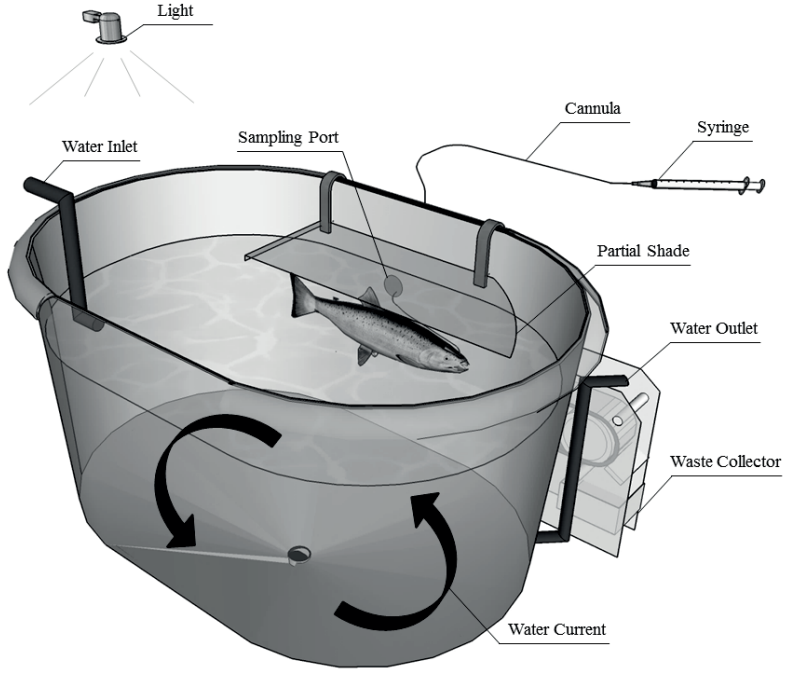


Figure 4. Illustration of the tank design, where the position of the light, shade and water outlet directed the dorsal aorta-cannulated rainbow trout adjacent to a sampling port for undisturbed blood collection.

In Papers IV and V, 2mL of blood were collected via caudal vein puncture from the tail of the fish after sedation using a heparinised syringe (Figure 5).

Figure 5. Blood collection from rainbow trout by (left) dorsal-aorta cannulation, as used in Papers I-II, and (right) caudal vein puncture, as used in Paper V.

Blood collected from cannulated fish in Papers I and II was analysed for sodium (Na), potassium (K), glucose, pH, partial carbon dioxide (PCO_2), total carbon dioxide (TCO_2), bicarbonate (HCO_3), base excess (BE) and haemoglobin (Hb) using EC8+ cassettes inserted into an i-STAT portable blood analyser (i-STAT Co, East Windsor, NJ, USA). Haematocrit (Hct) and leucocrit (Leu) were measured by microtube centrifugation. Red blood cell (RBC) counts were determined by 1:20 dilution with Turk's solution in a haemocytometer chamber and cells were counted in five squares at 400x magnification. Blood smears were stained with Giemsa and visualised with Nikon imaging software (Nikon Instruments Europe BV, Amsterdam, Netherlands) to assess RBC size. The Hb, Hct and RBC values were used to calculate mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) according to Stoskopf (1993) (see Section 2.5 for RBC index calculations). Blood was centrifuged and plasma was analysed for cortisol using multi-species Enzyme-Linked Immunosorbent Assay (ELISA) kits (DetectX®, Arbor Assays, Ann Arbor, MI, USA).

In Papers IV and V, the same methods were used to measure Hct, plasma cortisol and RBC indices. Otherwise, Hb was measured with a different method that used an initial reaction with ferric cyanide followed by analysis with a UV spectrophotometer (Stoskopf, 1993). The RBC counts and size were determined by a 1:100 dilution with Natt-Harrick's solution in a haemocytometer and images were assessed for both RBC count and size. Plasma glucose was measured after initial reaction with hexokinase and G6P-dehydrogenase, followed by UV spectrophotometry (R-Biopharm AG, Darmstadt, Germany). The pH of blood and content from the distal gut were analysed using an Orion ROSS™ micro-electrode and Orion Star™ pH meter (Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.4.2 Diet and gut yeast

Fish in Papers III and IV were dissected and the distal gut was removed under aseptic conditions. In Paper III, the gut was cut open and the gut content and the mucosa were scraped together into sterile tubes, while in Paper V the content and mucosa were collected separately. In both papers, diet and gut samples were analysed for yeast by serial dilution with bactopeptone and Tween 80 and plating on yeast peptone dextrose (YPD) agar supplemented with chloramphenicol, according to Petersson *et al.* (1999). To plate the samples, the streaking method with 100µL was used in Paper III, while the micro-droplet method with 10µL was used in Paper IV to save time and resources. The YPD plates were incubated at 25°C for 2-4 days. Plates that contained between 10 and 100 colonies were counted and multiplied by the dilution factor to obtain CFU g^{-1} of sample, referred to as yeast load or live yeast (Figure 6).

To identify yeasts, 10 isolates from YPD plates were re-isolated on YPD and incubated as before. The DNA was extracted, PCR-amplified and Sanger-sequenced according to Olstorpe *et al.* (2008). In brief, a loop of each isolate was denatured in NaOH at 95°C and PCR-amplified using PuReTaq Ready-To-Go™ PCR Beads (GE Healthcare Life Sciences, Uppsala, Sweden) and NL1-NL4 primers that targeted the D1/D2 region of the 26S rRNA gene. Amplicons were purified and sequenced using Sanger sequencing at Macrogen Inc. (Amsterdam, Netherlands) and then identified using BLAST® software and the nucleotide database resources of the National Center for Biotechnology Information (NCBI). This identification method was used in Papers III and V, but the yeast isolates were first identified using MALDI-TOF MS in Paper V. However, MALDI-TOF MS was unable to identify all the yeast isolates except for *S. cerevisiae*. This method involved a loop of yeast extracted with formic acid, acetonitrile and ethanol and then plotted on Biotarget plates with matrix solution and inserted into a Benchtop MicroFlex LT/SH MALDI-TOF MS using Compass software (Bruker Daltonics GmbH, Bremen, Germany).

Counts of yeast cells were determined by serial dilution of diets and yeast ingredients and staining with trypan blue. Yeast cells were counted in a haemocytometer under 400x magnification, according to the manufacturer's manual (Sigma-Aldrich Co). Cells that were infiltrated by blue stain were considered non-viable and were compared against viable cells without stain infiltration to determine percent viability (Figure 6).

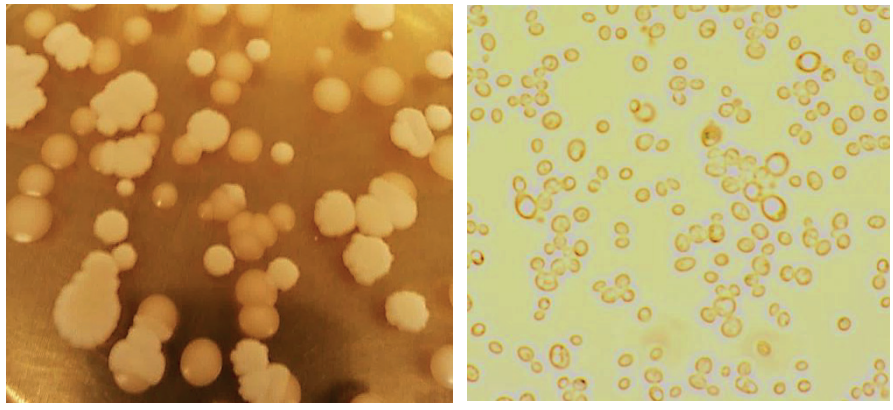


Figure 6. Yeast counted (left) on agar plates (1x) and (right) under the microscope (400x).

2.4.3 Diet and gut bacteria

In Papers III and IV, diet and gut samples were serially diluted with 0.9% NaCl and plated on tryptic soy agar (TSA) by the micro-droplet method. Counts were multiplied by the dilution factor to obtain CFU g^{-1} of sample, referred to as bacterial load or live bacteria.

Gut materials in Papers III and IV, and diets in Paper IV, were extracted for DNA, PCR-amplified with barcodes, purified with magnetic beads, pooled into a single library and sequenced using an Illumina MiSeq platform according to Herlemann *et al.* (2011) and Hugerth *et al.* (2014). In brief, samples were homogenised using silica beads and DNA was extracted using QiaAmp mini-kits (Qiagen Gmbh, Hilden, Germany). The PCR reactions consisted of extracted DNA, Phusion® High-Fidelity Master Mix (Thermo Fisher Scientific Inc., Waltham, MA, USA) and 515F and 805R Illumina primers that targeted the V4 region of 16S rRNA gene. Amplicons were PCR-amplified again in order to individually barcode each sample and then purified with AMPure XP® magnetic beads (Beckman Coulter Inc., Bromma, Sweden). Samples were quantified using a Quibit 3.0 Fluorometer (Invitrogen, Thermo Fisher Scientific), diluted to approximately 10nM and pooled into a single library. The library was sequenced using the MiSeq Illumina platform at SciLifeLab AB (Stockholm, Sweden).

Illumina data were trimmed to remove adapters, primers, low quality reads and long reads using Python software (Python Software Foundation, 2017), according to (Martin, 2011). Paired ends were joined using In Quantitative Insights into Microbial Ecology (QIIME) software (Caporaso *et al.*, 2010). Operational taxonomic units (OTUs) were assigned using the open reference

OTU-picking strategy at a threshold of 97%, using U-CLUST against the Greengenes database and taxanomy was assigned using Ribosomal Database Project (RDP) with a minimal threshold of 80%. Each OTU had to be present in at least three samples and were excluded if identified as chloroplasts and mitochondria (present in plant and fishmeal ingredients), since only bacteria were of interest.

α-diversity of bacterial OTUs was determined using the Shannon and Chao-1 richness indices with Paleontological Statistics (PAST) software (Hammer *et al.*, 2001). β-diversity of bacterial OTUs was determined using principal component analysis (PCA) in Paper III and non-metric dimensional scaling (NMDS) with Bray-Curtis similarity index in Paper V with PAST software.

2.4.4 Intestinal gene expression

In Paper V, gene expression of inflammatory cytokines and heat shock proteins was analysed using qPCR with SYBR Green dye after mRNA extraction and cDNA synthesis, according to Niklasson *et al.* (2014). In brief, proximal and distal gut regions were removed from three fish per tank and the gut content was squeezed out and discarded. A scalpel was used to collect the gut mucosa from each region into tubes of RNAlater® (Sigma-Aldrich AB), which were later stored at -80°C. The mucosa was homogenised using metal bead beating and mRNA was extracted using RNeasy® Plus Mini kits (Qiagen NV). A Nanodrop was used to determine the quantity of mRNA and each sample was diluted to 1000ng. The cDNA was synthesised using iScript™ Synthesis kits (Bio-Rad Laboratories Inc., Copenhagen, Denmark) and PCR amplification. Reactions of cDNA, SYBRGreen Supermix (Bio-Rad Lab Inc.) and primers were performed in the CFX Connect Real-time PCR Detection System (Bio-Rad Lab Inc.). Primers targeted genes corresponding to interferon-γ (IFNγ), tumour necrosis factor-α (TNFα), transforming growth factor-β (TGFβ), interleukin-1β/8/10/17 (IL-) and heat shock protein 70/90 (HSP-). Expression of each target gene was calculated relative to the reference gene β-actin based on its threshold cycle, (C_T) using equation $2^{-(C_T(\text{target})-C_T(\text{reference}))}$ based on the $2^{-\Delta C_T}$' method (Livak & Schmittgen, 2001).

2.5 Calculations

Growth performance was reported in all five papers, even though it was not the main objective. Thermal growth coefficient (TGC; only in Papers IV and V), specific growth rate (SGR), weight gain (WG), feed intake (FI) and feed

conversion ratio (FCR) were calculated using values of initial body weight (IBW) and final body weight (FBW):

$TGC = 100 \times [(FBW^{1/3} – IBW^{1/3}) \times (temperature \times days)^{-1}]$

$SGR (\%\ BW\ day^{-1}) = 100 \times [(\ln FBW – \ln IBW) \times D^{-1}$

$WG\ \% = 100 \times [(FBW – IBW) \times IBW^{-1}]$

$FI (g\ DM) = [(Feed\ given – Feed\ waste) \times recovery^{-1}] \times Feed\ given^{-1}$

$FCR = FI \times WG^{-1}$

In Paper V, body organ weight of the liver (LW) and viscera (VW) were used to calculate viscerosomatic index (VSI) and hepatosomatic index (HSI):

$VSI\ \% = 100 \times (VW \times FBW^{-1})$

$HSI\ \% = 100 \times (LW \times FBW^{-1})$

In Papers I and V, blood measurements of Hct, Hb and RBC were used to calculate RBC anaemia indices of MCV, MCH and MCHC:

$MCV (fL) = 10 \times (Hct \times RBC^{-1})$

$MCH (pg) = 10 \times (Hb \times RBC^{-1})$

$MCHC (g\ dL^{-1}) = 100 \times (Hb \times Hct^{-1})$

2.6 Statistical analyses

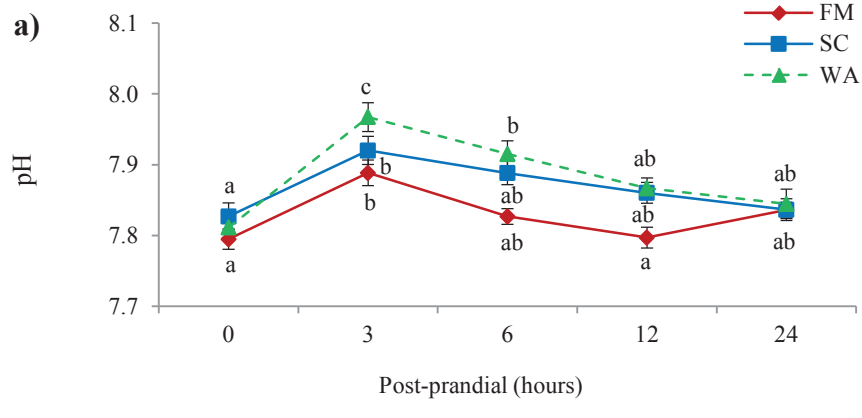
Different statistical models were used in each experiment (I-II, III and IV-V) since different experimental designs (*e.g.* cross-over or factorial) were implemented, although some aspects were common to all papers. In Papers I and II, significant differences between diets and sampling points were determined using Linear Mixed Effects models (LME; Lme package) in R® statistical software (R-Core-Team, 2015; Pinheiro *et al.*, 2014) followed by *post hoc* analysis using Least Square Means (Lsmeans package) test with Tukey adjustment. Fixed effects included in the LME were diet, sampling time, feed intake and fish weight, random effects of fish and week, interaction between diet and hour, and correlation between hour and fish-week.

In Paper III, Linear Models (LM) in R® software was used instead of LME with the fixed effect of tank. In Papers IV and V, differences were determined by LME that included diet and temperature as fixed effects, the interaction between diet and temperature and the random effect of tank. Papers III-V used each set of models to determine differences between treatments for microbial loads, diversity and OTUs with >1% abundance. For microbial profiles, differences between treatments were determined using Similarity Percentage Analysis (SIMPER) followed by Analysis of Similarity (ANOSIM) with Bray-Curtis similarity index in PAST software.

3 Main results

3.1 Whole blood effects of feeding inactivated yeasts (Paper I)

Rainbow trout fed in a cross-over experiment for three weeks with diets that replaced 60% fishmeal protein with *S. cerevisiae* and *W. anomalus* yeast showed increased levels of blood pH, TCO_2, HCO_3, BE and MCH compared with fish fed the fishmeal diet (Figure 7a). The fishmeal ingredient had higher ash content and buffering capacity than the yeast ingredients. Fish fed *W. anomalus* also had reduced RBC size. After the fourth week, no diet-stress interaction was found for fish fed yeast or fishmeal diets followed by netting for 1 minute, as plasma cortisol and glucose increased but returned to baseline levels within 24 hours (Figure 7b).



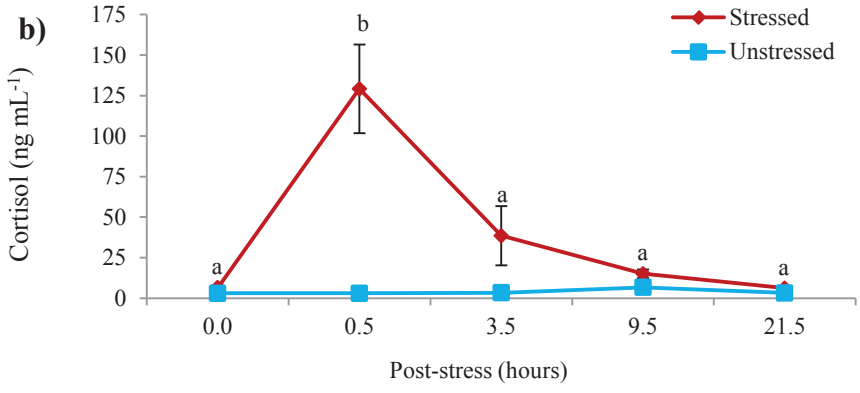


Figure 7. Values (mean ± standard error) of a) blood pH and b) plasma cortisol in rainbow trout fed diets containing fishmeal (FM), *S. cerevisiae* (SC) or *W. anomalus* with *S. cerevisiae* (WA). Different letters indicate significant difference at $p<0.05$.

3.2 Plasma amino acid effects of feeding yeast (Paper II)

Rainbow trout fed for three weeks with diets that replaced 60% fishmeal with *S. cerevisiae* and *W. anomalus* yeast showed similar plasma profiles of essential, non-essential and proteinogenic amino acids. However, methionine and sarcosine were significantly higher in fish fed the yeast diets (Figure 8), while hydroxy-proline and 3-methyl-histidine concentrations were reduced. After the fourth week, no diet-stress interaction was found for fish fed the yeast or fishmeal diets following a netting-stressor, but stressed fish had significantly higher levels of taurine and cystathionine than unstressed fish.

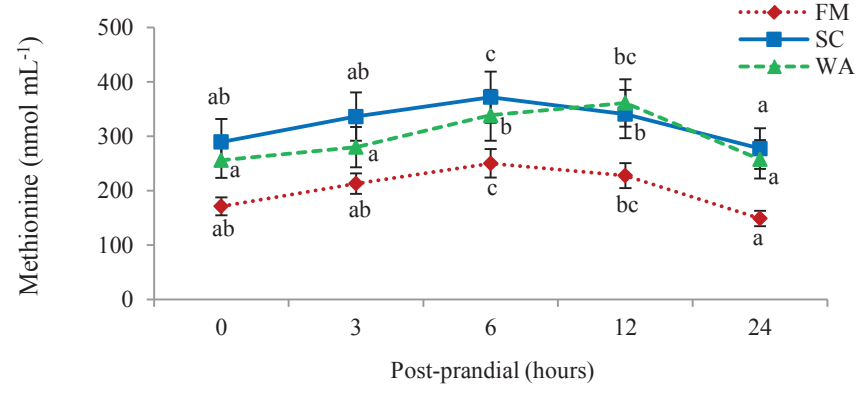


Figure 8. Plasma methionine concentration (mean ± standard error) in rainbow trout fed diets containing fishmeal (FM, ♦), *S. cerevisiae* (SC, ■) or *W. anomalus* with *S. cerevisiae* (WA, ▲). Different letters indicate significant difference at $p<0.05$.

3.3 Gut microbiota effects of feeding inactivated yeast (Paper III)

Heat extrusion reduced the yeast load in the yeast diets by 5-7 log CFU g^{-1} to levels similar to the load in the fishmeal diet with no dietary yeast. After 10 weeks of feeding graded levels of yeast to rainbow trout, yeast load in the distal gut did not change, except for SC40, and was dominated by *Debaryomyces hansenii*, with small inclusions of *Candida*, *Rhodotorula* and *Saccharomyces* species (Figure 9a). For gut bacteria, diversity and abundance were not affected by *S. cerevisiae* inclusions, while 40 and 60% replacement with *W. anomalus* mix significantly decreased diversity and altered abundance. Most sequences of bacteria were classified in phyla Firmicutes and Proteobacteria, specifically sequences identified as Leuconostocaceae, Lactobacillaceae and *Photobacterium* (Figure 9b).

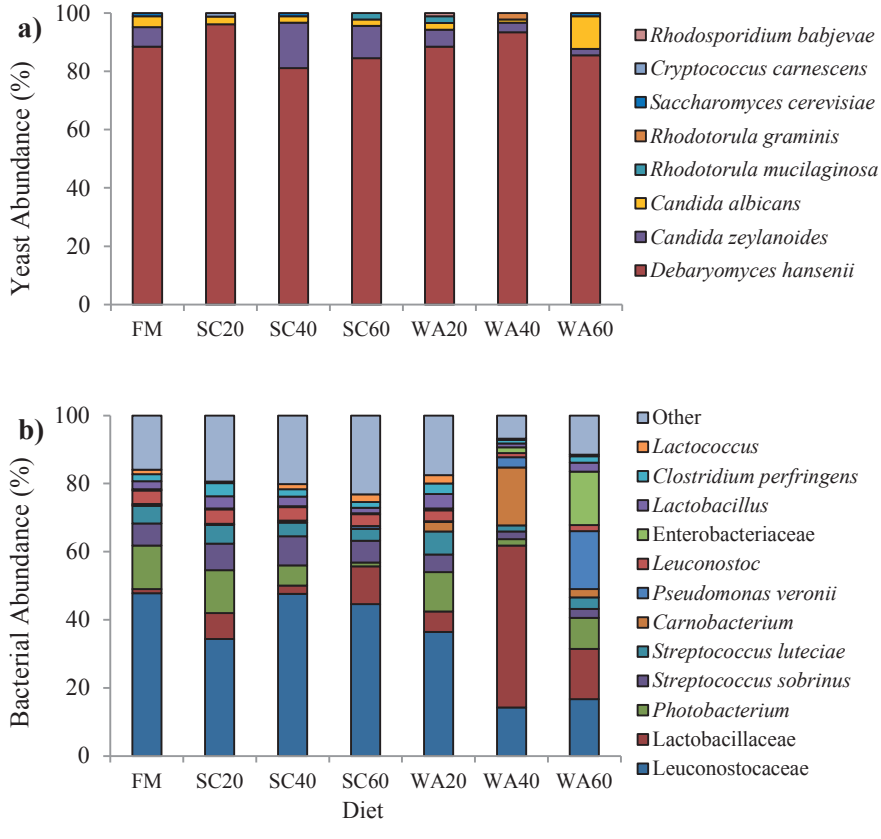


Figure 9. Mean relative abundance of a) live yeast and b) bacterial OTUs in the distal gut of rainbow trout fed diets containing fishmeal (FM) or 20, 40 and 60% replacement of fishmeal with *S. cerevisiae* (SC) or *W. anomalus* with *S. cerevisiae* (WA).

3.4 Gut microbiota effects of live yeast and temperature (Paper IV)

Cold pelleting reduced the yeast load in the yeast diet by 1-2 log CFU g^{-1}, while no detectable level of live yeast was found in the fishmeal diet. After six weeks of feeding live yeast, yeast loads in the gut content and mucosa were 4-5 log CFU g^{-1} higher in fish fed yeast than fishmeal. All yeasts in the gut of fish fed yeast were identified as *S. cerevisiae*, while fish fed fishmeal contained *Saccharomyces*, *Rhodotorula* and *Debaryomyces* species (Figure 10a). For gut bacteria, diversity was lower in fish reared in warm than cold water, except when fed yeast. Diversity was also lower in the gut mucosa than the gut content. Most sequences of bacteria were classified in phyla Tenericutes, Proteobacteria and Firmicutes, specifically sequences identified as Mycoplasmatales, *Mycoplasma* and *Pseudomonas veronii* (Figure 10b). Fish fed fishmeal while kept in cold water had higher abundance of lactic acid bacteria, especially Leuconostocaceae, and *Photobacterium*.

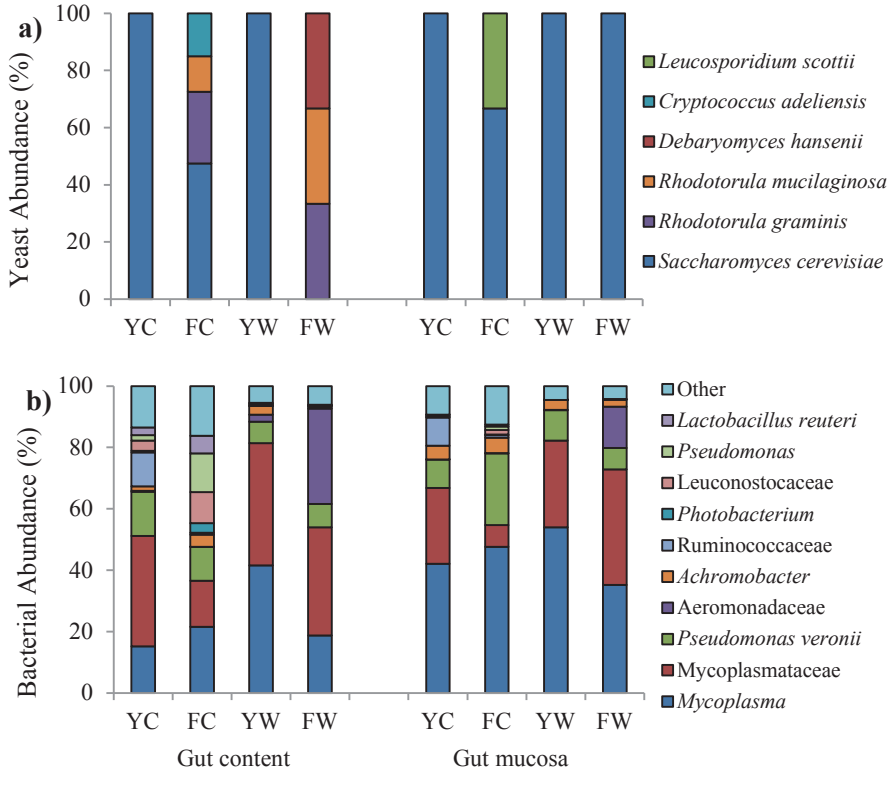


Figure 10. Mean abundance of a) yeast and b) bacteria in the gut content and mucosa of rainbow trout fed diets containing yeast (Y) or fishmeal (F) and kept in cold (C) or warm (W) water.

3.5 Whole blood and immune effects of live yeast and temperature (Paper V)

After six weeks of feeding live yeast, growth performances were similar except for lower TGC in fish kept in warm water. Temperature had a significant effect on Hct and RBC counts and resulted in significantly higher Hb and MCHC levels in fish in warm water (Figure 11a). Temperature also had a significant effect on plasma cortisol concentration, which was numerically increased in fish kept in warm water. Both blood and gut pH decreased in fish fed fishmeal in warm water, while pH was similar in fish fed yeast (Figure 11b). In the proximal gut, feeding yeast suppressed expression of pro-inflammatory cytokines TNFα and IL8, while increased temperature suppressed expression of pro- and anti-inflammatory cytokines IFNγ, IL8, IL17 and TGFβ (Figures 11c and 11d). In the distal gut, no significant changes in expression were found between treatments, although there was a significant diet-temperature interaction for IL1β and a temperature effect for HSP90.

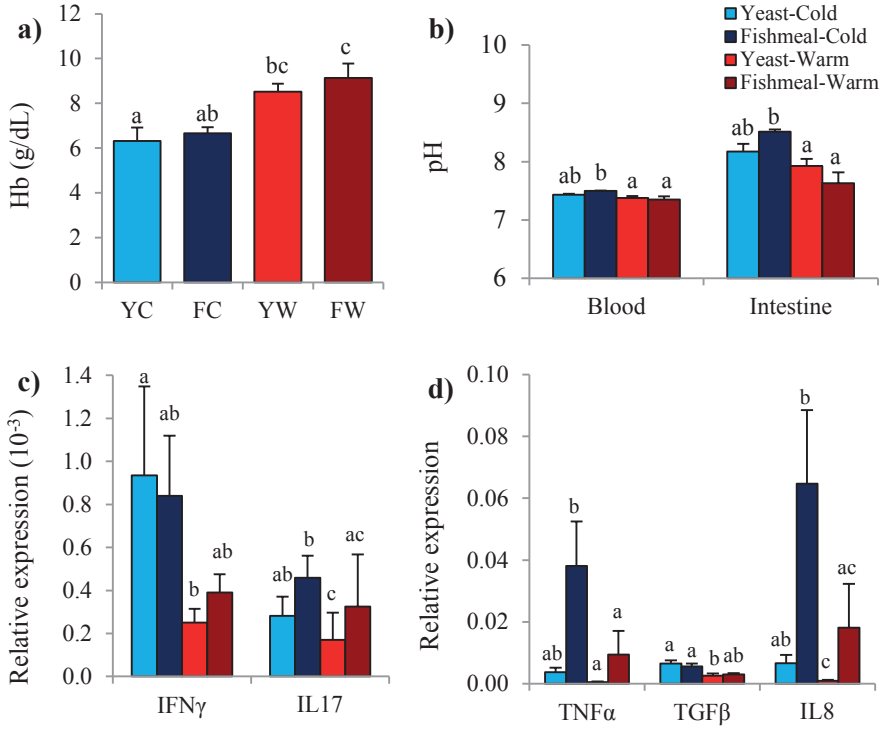


Figure 11. Values (mean ± SE) of a) blood Hb, b) pH and c) and d) gene expression of cytokines relative to β-actin in the gut of rainbow trout fed diets containing yeast (Y) or fishmeal (F) and kept in cold (C) or warm (W) water. Different letters indicate significant difference at $p<0.05$.

4 General discussion

The main results of this thesis were compared with those of previous studies, in order to gain a better understanding of the mechanisms underlying the effects of feeding inactivated and live yeast on blood physiology and gut microbiota of rainbow trout observed in Papers I-V.

4.1 Fish growth on yeast diets

Evaluation of fish growth was not the main aim of this thesis, but reduced growth is a tertiary indicator of stress and can help explain possible dietary effects on fish health. In Paper III, the results were compared against those of a parallel study that evaluated fish growth after feeding graded levels of inactivated yeasts, *S. cerevisiae* and *W. anomalus* to rainbow trout for 10 weeks (Vidakovic *et al.*, submitted). The results of that study indicated that fish fed 60% replacement of fishmeal protein with yeast had reduced growth performance, while no differences were found at 40% replacement (Figure 12). These results agree with previous findings that yeast can replace up to 40% fishmeal without negative effects on growth performance of salmonids (Vidaković *et al.*, 2016; Sealey *et al.*, 2015; Hauptman *et al.*, 2014; Øverland *et al.*, 2013). Based on these findings, fish in Papers IV and V were fed live yeast that replaced 40% of fishmeal protein and no significant differences in SGR were found compared with fish fed fishmeal at both water temperatures tested. Interestingly, SGR of fish fed yeast while kept in warm water (18°C) was numerically higher than that of fish fed yeast and kept in cold water (11°C), which suggests that live yeast diets may be more digestible at warmer temperatures. Previous studies have found that higher water temperature increases protein digestibility for rainbow trout (Yamamoto *et al.*, 2007), but this may have a higher impact on fish fed yeast diets since yeast has been found to be less digestible than fishmeal for salmonids (Vidakovic *et al.*,

submitted; Vidaković *et al.*, 2016; Øverland *et al.*, 2013). Moreover, it was surprising that fish growth was similar for fish fed fishmeal and those fed live yeast, since intact yeast cells have been found to reduce fish growth compared with disrupted yeast cells (Rumsey *et al.*, 1990).

Lower TGC of fish kept in warm water indicates that 18°C may have been above their optimal growth temperature. Previous studies have reported that the optimal growth temperature for rainbow trout is between 13 and 17°C (Bear *et al.*, 2007; Austreng *et al.*, 1987; Wurtsbaugh & Davis, 1977). Therefore, 18°C was too high and resulted in similar fish growth to the below-optimal temperature, *i.e.* 11°C. Fish fed live yeast and kept at 11°C had the lowest growth, although not significant, but the significant effect of diet indicates potential problems if live yeast was fed in a long-term experiment. Compared with live yeast, 40% replacement of fishmeal protein with inactivated yeast (Paper III) may be a better option to maintain fish growth, but side-by-side comparisons are needed. Overall, Papers III and V showed that replacement of 40% fishmeal protein with inactivated and live yeast did not significantly reduce growth of rainbow trout and that higher water temperature may result in increased digestion of yeast cells.

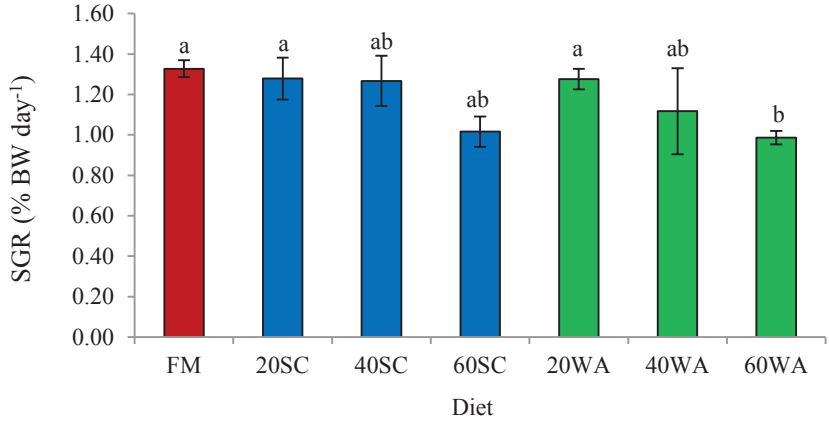


Figure 12. Specific growth rate (SGR; mean ± standard error) of rainbow trout in Paper III fed diets containing fishmeal (FM) or 20, 40 and 60% replacement of fishmeal with *S. cerevisiae* (SC) or *W. anomalus* with *S. cerevisiae* (WA). Calculations based on data from (Vidakovic *et al.*, submitted). Different letters indicate significant difference at $p<0.05$.

4.2 Buffering capacity of yeast

The elevated levels of blood pH, TCO_2, HCO_3 and BE in Paper I clearly indicate that yeast diets induced a higher alkaline tide than the fishmeal diet

(see Figure 7a). The yeast ingredients were found to have lower ash content and buffering capacity than fishmeal (Figure 13), characteristics that have been shown to increase alkaline tide in rainbow trout (Bucking & Wood, 2008; Cooper & Wilson, 2008). Alkaline tide is caused by an efflux of HCO_3 into the blood to counteract the secretion of HCl into the stomach in order to maintain the acid-base balance during digestion (Niv & Fraser, 2002). In contrast, in Paper V fish fed fishmeal and reared in cold water had the highest blood pH. This may be explained by the difference in diet production method and water temperature, since diets in Paper I were heat-extruded and fed at 15°C and those in paper V were cold-pelleted and fed at 11°C. Heat extrusion has been shown to increase diet digestibility in fish compared with cold pelleting (Stone *et al.*, 2005) and activity of metabolic enzymes correlate with temperature (Azevedo *et al.*, 1998). This suggestion is supported by the decrease in blood pH for fish fed fishmeal, which suggests that increased water temperature resulted in increased diet digestibility and buffering capacity of the fishmeal diet. Increased digestibility of protein, lipids and starch at higher water temperatures has been shown in a previously (Yamamoto *et al.*, 2007). However, blood pH in fish decreases with increased water temperature due to off-loading of CO_2 by red blood cells (Jensen *et al.*, 1998). There was a significant effect of temperature, but only a tendency for an effect of diet on blood pH in Paper V. The significant diet-temperature interaction for gut pH is also difficult to explain and again indicates a possible diet-blood-gut-temperature connection. Overall, these results show that both dietary ingredients and water temperature play a role in blood/gut pH of rainbow trout.

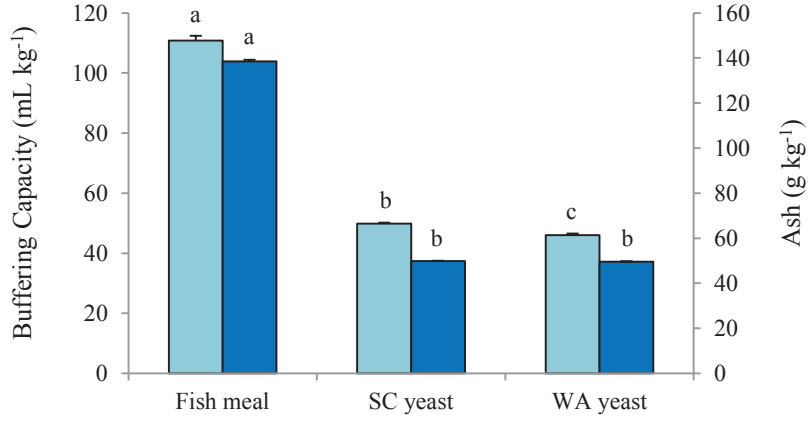


Figure 13. Values (mean ± standard error) of buffering capacity (light blue) and ash content (dark blue) of fishmeal, *S. cerevisiae* and *W. anomalus* with *S. cerevisiae* mix ingredients. Different letters for each parameter indicate significant difference at $p<0.05$.

4.3 Yeast-induced red blood cell anaemia

Fish fed heat-extruded yeast diets (60% fishmeal replacement) in Paper I showed increased MCH levels and smaller RBC, which are signs of hyperchromic anaemia (Figure 14). In contrast, fish fed cold-pelleted yeast diets (40% fishmeal replacement) in Paper V showed similar MCH levels and RBC size, except there was a tendency for an effect of diet. This comparison suggest that diets in which yeast replaces 40% of fishmeal protein instead of 60% do not induce red blood cell anaemia, although 40% is close. However, more yeast cells survived digestion (not metabolised) in Paper V when fish were fed live yeast, which may allow higher dietary inclusion of live rather than inactivated yeast. Previous studies have found that 100% replacement of fishmeal with live *W. anomalus* in diets fed to rainbow trout can result in changes in MCH levels and in RBC size and shape (Sanchez-Muniz *et al.*, 1982; Sanchez-Muniz *et al.*, 1979). Those authors suggested that rainbow trout are not capable of metabolising the high content of nucleic acid found in yeast since degradation results in high production of reactive oxygen species that oxidise haemoglobin to methaemoglobin, which impairs oxygen transport and RBC function form Heinz bodies that are eventually removed from the blood circulation (Jain, 1993). The content of nucleic acid was confirmed to be higher in the yeast ingredients than in fishmeal in Paper I. Therefore, unless nucleic acid content is reduced, the high level in yeast limits its replacement of fishmeal protein to a maximum of 40% in diets for rainbow trout.

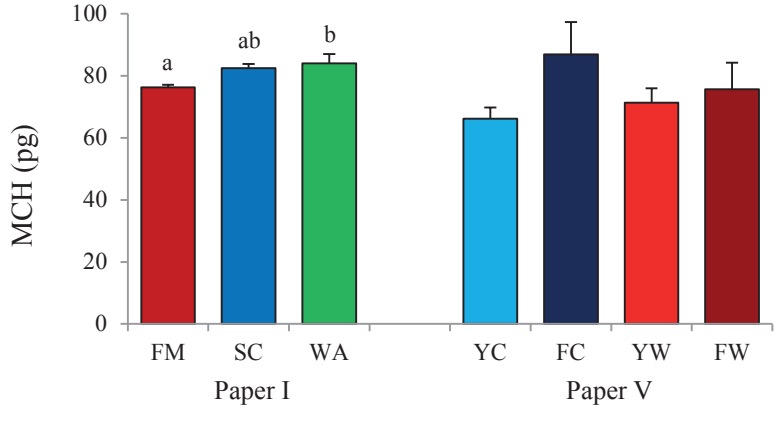


Figure 14. Mean corpuscular haemoglobin (MCH; mean ± standard error) of rainbow trout in Paper I fed diets containing fishmeal (FM), *S. cerevisiae* (SC) or *W. anomalus* with *S. cerevisiae* (WA) compared fish in Paper V fed yeast (Y) and fishmeal (F) and reared in cold (C) or warm (W) water. Different letters indicate significant difference at $p<0.05$.

4.4 Amino acid profiles of yeast and fish plasma

The similarities in post-prandial plasma amino acid profiles between fish fed yeast and fishmeal diets (Figure 15) suggest that reductions in growth performance of fish fed high inclusions of yeast may not be due to low content of amino acids in the yeast ingredients. The concentrations of individual essential and non-essential amino acids in the *S. cerevisiae* and *W. anomalus* mix yeast ingredients were generally lower than in fishmeal, but formulation of yeast diets with a mix of fishmeal and plant ingredients resulted in similar amino acid content in the yeast and fishmeal diets (see Paper II). This finding complements the previous suggestion that the high content of nucleic acids in yeast and the resulting haemolytic anaemia may be the main factor responsible for reduced fish growth.

Methionine is suspected to be a deficient in yeast and thus more crystalline methionine was supplemented in the yeast diets. Previous studies have shown that crystalline amino acids result in higher plasma amino acid peaks than non-supplemented diets fed to rainbow trout (Barrows *et al*., 2007; Schuhmacher *et al*., 1997; Yamada *et al*., 1981). The increased levels of plasma methionine and its derivative, sarcosine, found in Paper II (see Figure 8) suggest that supplementary methionine may not be needed. In a separate study using the same diets, rainbow trout fed a diet with 60% replacement of fishmeal with yeast had similar growth performance to fish fed yeast supplemented with methionine (Vidakovic *et al*., submitted). Previous studies have also found no effect of methionine supplementation on fish growth performance (Oliva-Teles *et al.*, 2006; Mahnken *et al.*, 1980), thus these findings suggest its inclusion in yeast diets is not necessary.

The higher levels of hydroxy-proline and 3-methyl-histidine (non-proteinogenic amino acids) in the fishmeal ingredient, and consequently the higher plasma levels in fish fed the fishmeal diet, illustrate that the plasma profile reflected the diet composition. However, supplementation of hydroxy-proline has been shown to increase growth of Atlantic salmon (Aksnes *et al.*, 2008). Instead of methionine, hydroxy-proline may be a better candidate for supplementation in diets that replace fishmeal with yeast, but more research is needed. Furthermore, the similar amino acid profiles in stressed fish fed yeast and fishmeal diets indicate that the amino acid content of the yeast diets was sufficient in eliciting an acute stress response.

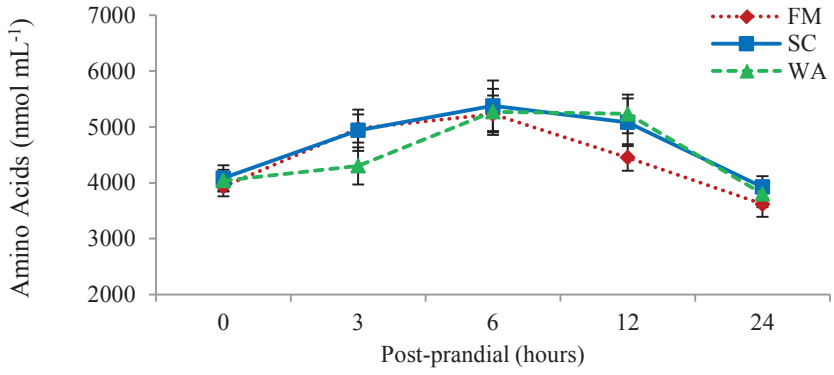


Figure 15. Total plasma amino acid concentration (mean ± standard error) in rainbow trout fed diets of fishmeal (FM, ♦), *S. cerevisiae* (SC, ■) or *W. anomalus* with *S. cerevisiae* (WA, ▲).

4.5 Feed processing and yeast content in the diet

The low load of live yeast cultured on agar and the presence of large numbers of intact yeast cells counted under the microscope indicated that heat-extrusion inactivated the majority of yeast cells in Paper III (Figure 16). However, the yeasts were determined to be viable, but not culturable, following extrusion. In comparison, the high load of live yeast and counted yeast cells in Paper IV indicated that cold pelleting did not inactive a large load of yeast. Overall, heat-extrusion resulted in 5-7 log reductions in live yeast, compared with only 1-2 log reductions with cold-pelleting. Therefore, heat extrusion inactivated yeasts, enabling dietary inclusion of yeast mainly as a protein source, whereas the majority of yeast cells survived the cold-pelleting process and these diets were able to be tested as both protein and probiotic sources.

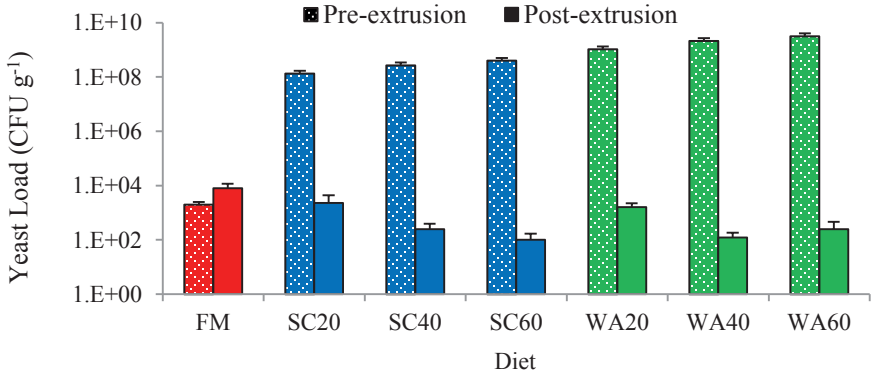


Figure 16. Yeast load (mean ± standard error) in diets in Paper III of fishmeal (FM) or 20, 40 and 60% replacement of fishmeal with *S. cerevisiae* (SC) or *W. anomalus* with *S. cerevisiae* (WA).

The abundance of yeast species in the diets drastically changed following heat-extrusion of the diets in Paper III. In raw ingredients, species of *Saccharomyces*, *Debaryomyces*, *Wickerhamomyces*, *Candida*, *Hyphopichia*, *Cystofilobasidium*, *Cryptococcus*, *Sporobolomyces* and *Trichosporon* were present, while after extrusion only the added yeasts, *S. cerevisiae* and *W. anomalus*, survived. In Paper IV, the non-yeast ingredients were not analysed for yeast composition, but after cold-pelleting only *S. cerevisiae* was present in the yeast diet, while no yeast was detected in the fishmeal diet. The discrepancy between the 3-4 log CFU g^{-1} found in the fishmeal diet in Paper III compared with no detectable yeast (below 2 log CFU g^{-1} detection limit for 1:100 dilution) found in the fishmeal diet in Paper IV may be due to the different locations, equipment and ingredients used. The diets in Paper III were produced at the Natural Resources Institute in Finland, where diets are made regularly and stored at room temperature, while the diets in Paper IV were produced at the SLU Feed Science Laboratory in Sweden with disinfected equipment, where diets and ingredients are stored at 5°C. It is difficult to determine the exact cause of the increased yeast load in the diets in Paper III, given the numerous differences in diet preparation and production characteristics. In addition, plant-based diet ingredients can be contaminated with fungi, such as yeasts and moulds, during development, harvest and storage, especially when optimal conditions for fungal growth prevail (Forbes *et al.*, 1992). The results in Papers III and IV indicate that yeast load and abundance in the diet may vary depending on diet production preparation and production methods.

4.6 Dietary yeast and gut yeast

The yeast load in the gut of fish fed fishmeal was much higher in Paper III (*i.e.* 5-6 log CFU g^{-1}) than in Paper IV (*i.e.* 2-3 log CFU g^{-1}), but fish fed yeast in Paper IV had the highest yeast load (*i.e.* 6-8 log CFU g^{-1}) (Figure 17). Previous studies have shown that yeast load in the gut can range from undetectable to 7 log CFU g^{-1} (Gatesoupe, 2007), and thus fish fed yeast in Paper IV had a higher yeast load than previously reported. The high load of gut yeast in fish in Paper IV can be directly attributed to the high yeast load in the diet, but the load in fish fed fishmeal in both papers should have been similar. One explanation could be the difference in water source, as fish in Paper III were supplied with surface water from a river, while fish in Paper IV were given groundwater. The yeast load in the water was below the detection limit in Paper IV, while the water was not analysed for yeast in Paper III. However, yeasts are commonly found in aquatic environments (Navarrete & Tovar-Ramírez, 2014) and the

lack of disinfection of intake water in Paper III would have exposed the fish to yeast and other microbes from the river. Colonisation of the fish gut with yeast via the water has been demonstrated previously (Gatesoupe, 2007). Therefore, the impact of the water source on yeast load in the gut should be considered in future yeast-related studies.

The abundance of yeast species in the gut was mostly unchanged after feeding graded levels of inactivated yeast in Paper III, while inclusion of live yeast in Paper IV had significant effects on gut yeast composition. The gut of fish in Paper III was dominated by *Debaryomyces hansenii* and small amounts of *Candida*, *Rhodotorula*, *Cryptococcus* and *Saccharomyces* species, while the gut of fish in Paper IV was dominated by *S. cerevisiae* and small amounts of the aforementioned species. The identification of similar yeast species in the gut of different populations of fish reared in separate locations in Kälarne and Uppsala, Sweden, indicates that these yeast species are part of the 'normal' gut microbiota of rainbow trout and agrees with previous studies (Gatesoupe, 2007). However, the abundance of *D. hansenii* in the gut was very low in Paper IV, whereas it is usually the dominant yeast found in the fish gut (Navarrete & Tovar-Ramírez, 2014). Again, the dominance of *S. cerevisiae* and low abundance of *D. hansenii* may be due to the low yeast load in the groundwater in Paper IV. The dominance of *S. cerevisiae* in Paper IV may indicate a more 'normal' yeast composition in the gut of rainbow trout since water source had less of an impact.

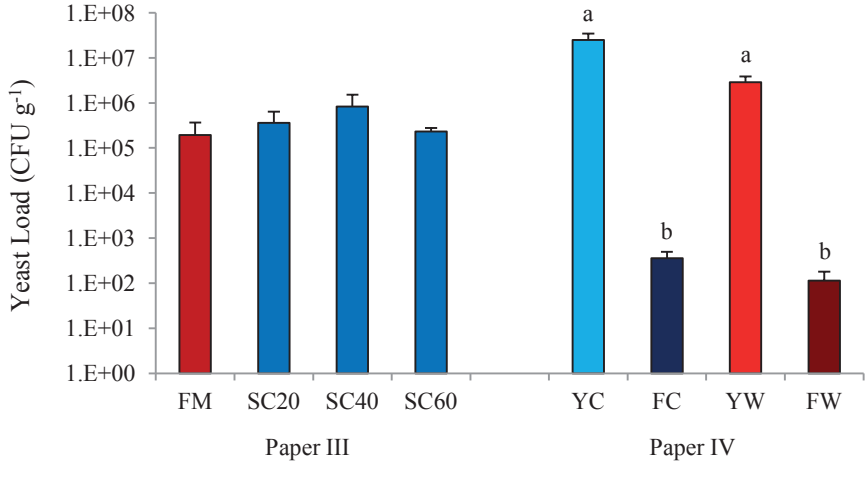


Figure 17. Yeast load (mean ± standard error) in the gut of rainbow trout fed fishmeal (FM) or 20, 40 and 60% replacement of fishmeal with inactivated *S. cerevisiae* yeast (SC) in Paper III compared with in the gut content of fish fed fishmeal (F) or live yeast (Y) while kept in cold (C) or warm (W) water in Paper IV. Different letters indicate significant difference at $p<0.05$.

4.7 Gut bacteria influenced by dietary yeast

The bacterial loads in the fish gut were similar in Papers III and IV, but again were slightly higher in Paper III than Paper IV and unaffected by temperature (Figure 18). Previous studies using culture-based methods have found that the bacterial load in the gut of rainbow trout varies between 4 to 8 CFU g^{-1} (Waché *et al.*, 2006; Aubin *et al.*, 2005), which reflects findings in this thesis. In addition, bacterial load in Atlantic salmon have been reported to remain the same when water temperature increased (Neuman *et al.*, 2016; Hovda *et al.*, 2012). In contrast, previous studies have found that bacterial load increases in the fish gut with warmer water temperatures (Naviner *et al.*, 2006; Al-Harbi & Uddin, 2004; Hagi *et al.*, 2004). Also, Aubin *et al.* (2005) found that feeding live yeast to rainbow trout reduced the bacterial load in the gut, whereas Paper III showed the opposite for fish fed diet SC40. It is difficult to compare the aforementioned studies since they used many different rearing conditions, diet formulations, sampling methods and/or fish species.

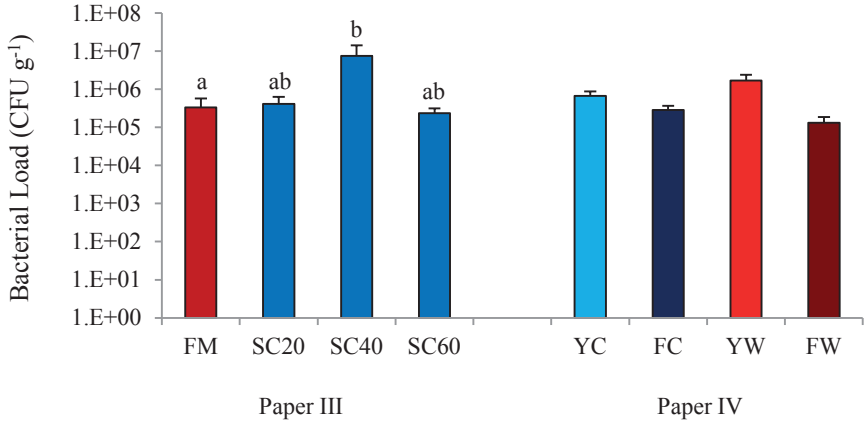


Figure 18. Bacterial load (mean ± standard error) in the gut of rainbow trout fed fishmeal (FM) or 20, 40 and 60% replacement of fishmeal with inactivated *S. cerevisiae* yeast (SC) in Paper III compared with Paper IV that fed fishmeal (F) or live *S. cerevisiae* yeast (Y) while kept in cold (C) or warm (W) water. Different letters indicate significant difference at $p<0.05$.

The overall abundance of bacteria in the gut of fish fed graded levels of inactivated *S. cerevisiae* was similar to that in fish fed fishmeal, but abundance and diversity were significantly affected when the fish were fed diets that replaced 40 and 60% with *W. anomalus* mix in Paper III (see Figure 9b). No differences in overall bacterial abundance were found between fish fed fishmeal and 40% replacement with live *S. cerevisiae* in Paper III, which agrees with Paper IV (Figure 19). In paper IV, increased water temperature reduced bacterial diversity, but not Shannon diversity for fish fed live yeast.

There was a significant interaction between diet and temperature, which suggests a potential benefit of feeding live yeast to maintain 'normal' bacterial diversity in the gut at warmer temperatures. Increased bacterial diversity in the gut of rainbow trout has been found when fed low inclusion of micro-algae, another SCP (Lyons *et al.*, 2016). These results indicate that feeding *S. cerevisiae* does not alter bacterial abundance or diversity in the gut of rainbow trout, except when fed inactivated *W. anomalus* or live *S. cerevisiae* at increased temperatures.

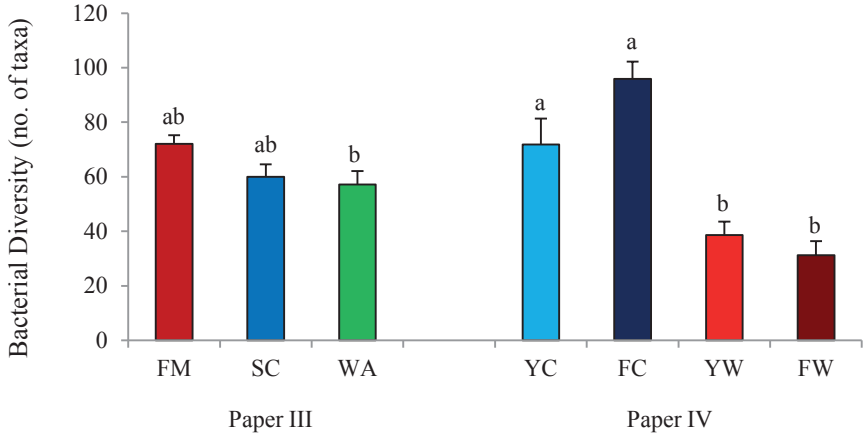


Figure 19. Bacterial diversity (mean ± standard error) in the gut of rainbow trout fed fishmeal (FM) or 40% replacement of fishmeal with inactivated *S. cerevisiae* yeast (SC) or *W. anomalus* mix (WA) in Paper III compared with Paper IV that fed fish fishmeal (F) or live yeast (Y) while kept in cold (C) or warm (W) water. Different letters indicate significant difference at $p<0.05$.

In Paper III, the gut bacteria were mainly represented by phyla Firmicutes and Proteobacteria, while in Paper IV the gut bacteria were dominated by Tenericutes and to a lesser extent Proteobacteria and Firmicutes. The reversal in the dominant bacteria phyla between the two papers may be related to the different diets or rearing conditions, and highlights the complexity of fish gut microbiota. Abundances of bacteria phyla were not significantly affected by diet in Paper III, whereas diet affected Tenericutes and Proteobacteria while temperature affected Tenericutes and Firmicutes in paper IV.

On the OTU level, Leuconostocaceae, *Lactobacillus*, *Photobacterium* and *Pseudomonas* were found in both papers with abundance >1% (Figure 20) and have been reported in previous studies that used high-throughput sequencing on gut microbiota of rainbow trout (Lyons *et al.*, 2017; Michl *et al.*, 2017; Lyons *et al.*, 2016; Lyons *et al.*, 2015). However, Paper IV had lower abundance of *Streptococcus* and *Lactococcus* (*i.e.* 0.01-0.1%) than reported in Paper III and previous studies. In Paper IV, *Lactobacillus* and *Photobacterium*

were highly abundant in both fishmeal and yeast diets (bacteria in the diets in Paper III were not determined) and corresponded to high abundance in the gut content, but not the gut mucosa.

Tenericutes, specifically *Mycoplasma*, have also been found in high abundance in the gut of rainbow trout in previous studies (Lyons *et al.*, 2017; Lyons *et al.*, 2016; Lowrey *et al.*, 2015) or not found at all (Michl *et al.*, 2017; Lyons *et al.*, 2015; Ingerslev *et al.*, 2014). *Mycoplasma* is a fragile bacterium that is small, lacks a cell wall and has strict nutrient requirements (Freundt & Razin, 1958), which suggests its presence is specific to certain aquatic environments, gut conditions and/or feed substrates. The high abundance of *Mycoplasma* in Paper IV and lack of abundance in Paper III suggests that the cold-pelleted diets fed to fish supplied with groundwater were more optimal for *Mycoplasma* gut colonisation than the heat-extruded diets fed to fish supplied with river water. However, fish in those papers were from different hatcheries and genetic families, which may have played a role in the abundance of *Mycoplasma*. More research is needed to understand the function of this bacterium and processes involved in its colonisation of the fish gut.

Lactic acid bacteria (order Lactobacillales) with >1% abundance in the gut in Papers III and IV included Leuconostocaceae, Lactobacillaceae, Enterobacteriaceae, *Leuconostoc, Lactobacillus, Streptococcus, Carnobacterium* and *Lactococcus* (Figure 20). In Paper III, abundance of Leuconostocaceae was significantly reduced in fish fed inactivated *W. anomalus*, but not *S. cerevisiae*, while in Paper IV Leuconostocaceae was reduced in fish fed live *S. cerevisiae* and kept in cold water. However, besides reduced abundance of Leuconostocaceae and *Photobacterium,* fish kept in cold water and fed live yeast or fishmeal were similar. In warm water, fish had significant reductions in lactic acid bacteria when fed either diet. Lactic acid bacteria have been found to be a beneficial group in the gut due to their antagonism toward pathogens and their ability to produce nutrients that can be absorbed and used by the fish (Ringø & Gatesoupe, 1998), thus increased temperature and feeding *W. anomalus* may not be beneficial to gut microbiota. Again, composition of gut microbiota is complex and much is unknown about the positive and negative interaction between the gut and microbes.

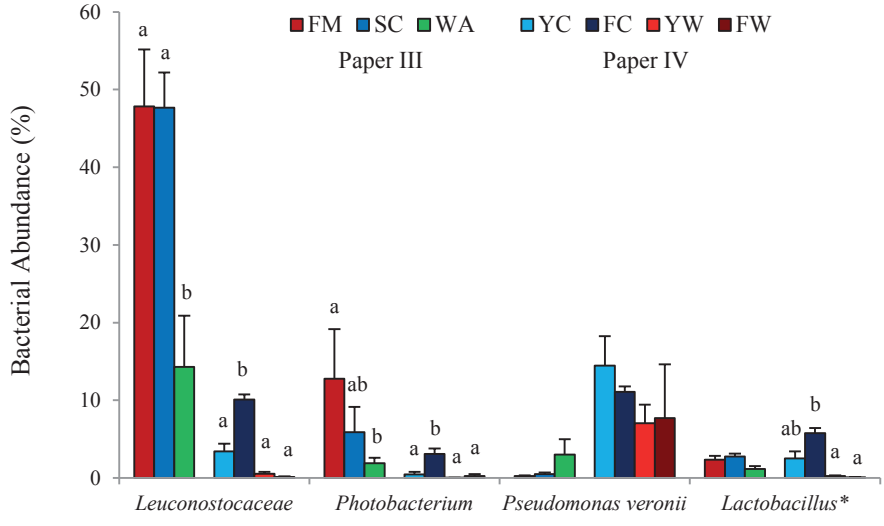


Figure 20. Relative bacterial abundance (mean ± SE) of common OTUs in the gut of rainbow trout fed fishmeal (FM) or 40% replacement of fishmeal with inactivated *S. cerevisiae* (SC) and *W. anomalus* mix (WA) in Paper III and in the gut content of fish fed fishmeal (F) or live *S. cerevisiae* (Y) while kept in cold (C) or warm (W) water in Paper IV. Different letters indicate significant difference at $p<0.05$. *indicates the OTU was *Lactobacillus reuteri* in Paper IV.

4.8 Inflammatory effects of yeast and temperature

The objective of the gene expression analysis in Paper V was to determine whether feeding live yeast together with increased water temperature enhanced or suppressed the innate immune function in the gut of rainbow trout. The results showed that fish fed live yeast had reduced expression of pro-inflammatory cytokines compared with fish fed fishmeal (see Figure 11 and Table 4). Previous studies have found that feeding live yeast reduces expression of pro- and anti-inflammatory cytokines in the gut of Nile tilapia fed high levels of soybean meal (Ran *et al.*, 2015) and reduces soybean meal-induced enteritis (SBMIE) in Atlantic salmon (Grammes *et al.*, 2013). These studies both suggest that yeast reduces intestinal inflammation by secreting metabolites that improve the efficiency of metabolic pathways and stimulating the secretion of antimicrobial peptides. However, fish in Paper V were not fed soybean meal, and thus their gut should not have been inflamed from SBMIE. Suppressed expression of pro-inflammatory cytokines may lead to reduced immune response and increased risk of disease. In addition, it was unexpected not to find lower HSP expression in the gut of fish fed live yeast, as previous studies have shown this effect with probiotics that improve gut health (Ran *et*

al., 2015; Liu *et al.*, 2013). Further research is required in order to determine whether reduced cytokine expression has positive or negative effects on the gut health and immune response of rainbow trout.

Increased water temperature resulted in higher plasma cortisol levels and reduced expression of pro- and anti-inflammatory cytokines, which suggests that the warm water triggered a stress response that suppressed the innate immune response. In salmonids, slight but chronic elevations in plasma cortisol have been implicated in depression of immune function and disease resistance (Maule *et al*., 1987; Pickering *et al*., 1987). In Atlantic salmon, Niklasson *et al*. (2014) found that cortisol implants reduced CD8α lymphocytes and suppressed IFN type 1 expression in the proximal gut. In sea bass, stress and cortisol treatment have been shown to increase susceptibility to parasitic infections (Saeij *et al*., 2003). At higher water temperatures, Atlantic salmon are more vulnerable to gut inflammation, appetite loss, reduced growth and mortality (Vikeså *et al*., 2017; Green *et al*., 2013; Urán *et al*., 2008). In addition, a significant effect of temperature on HSP90 was found in the distal gut, which also indicates increased cell damage and activation of repair pathways (Lund & Tufts, 2003; Fader *et al.*, 1994). These results suggest that increased water temperature suppresses the innate immune response, which may increase the risk of disease in rainbow trout reared at a water temperature of 18°C.

Table 4. *Effects of diet, temperature and diet-temperature interaction on relative gene expression in the proximal and distal gut of rainbow trout*

	IFNγ	TNFα	TGFβ	IL1β	IL8	IL10	IL17	HSP70	HSP90
Proximal gut									
diet	ns	**	ns	**	**	ns	ns	ns	.
temp	***	***	***	ns	***	ns	***	ns	ns
diet x temp	ns	ns	ns	ns	ns	.	ns	ns	ns
Distal gut									
diet	ns	ns	ns	ns	ns	.	ns	ns	ns
temp	.	ns	.	ns	.	ns	ns	ns	**
diet x temp	ns	ns	ns	*	ns	ns	ns	ns	ns

IFNγ: interferon-γ, TNFα; tumour necrosis factor-α, TGFβ: transforming growth factor-β, IL: interleukin, HSP: heat shock protein.
P-values from linear mixed effects models with fixed effects of diet, temperature and diet-temp plus random tank effect. Symbols ***, **, *, . and ns refer to p-values <0.001, <0.01, <0.05, <0.10 and not significant.

5 Conclusions and future perspectives

Based on the results of this thesis, it can be concluded that inclusion rate, species, viability and temperature of dietary yeast are key factors that affect blood physiology and gut microbiota of rainbow trout. Fish fed diets in which 60% of fishmeal protein was replaced with yeast induced haemolytic anaemia, diets of *W. anomalus* altered gut bacteria composition, diets of live yeast increased load of gut yeast and increased temperature reduced load of gut yeast. In contrast, only minor effects of feeding inactivated and live *S. cerevisiae* on load, diversity and abundance of gut bacteria were found. Uptake of plasma amino acids was also not a factor. Yeast viability in the diet was dictated by the processing method as cold-pelleting resulted in substantially higher load of live yeast compared with heat-extrusion. At increased water temperature, feeding live yeast had a beneficial effect on diversity of gut bacteria, but this may be negated as it also resulted in reduced expression of inflammatory cytokines and potential suppression of the innate immune function in the gut. Therefore, these results suggest that feeding yeast with high content of nucleic acids, species of *W. anomalus* and live *S. cerevisiae* at increased water temperatures (*e.g.* during summer) should be avoided.

Future perspectives based on the results of this thesis point to possible solutions and feeding strategies that may increase the research, development and commercial use of yeast as protein and probiotic sources in feeds for salmonids. Feeding live yeast to improve gut microbiota and to reduce gut inflammation from SBMIE may become important strategies in the future. More research is needed on the reduction of nucleic acid content and optimisation of production conditions of yeast diets in order to increase the inclusion and safety of feeding yeast to salmonids. At the moment, moderate inclusion of yeast with a mix of plant sources may be a sustainable alternative to fishmeal diets for farmed fish, an alternative which will be increasingly needed as the aquaculture industry continues to expand.

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Popular science summary

The growing human population has led to concerns about food shortages in coming years. Aquaculture, farming of fish and aquatic plants, may be a possible solution to address food shortages, as climate change and overfishing limit the availability of wild-caught fish for human consumption. However, aquaculture uses fishmeal derived from wild stocks as a protein source in feeds for salmon and trout. This practice has been criticised as unsustainable since it increases pressure on wild stocks and diverts food that humans can eat. Yeast (*Saccharomyces cerevisiae*), often used in baking and brewing, may provide a solution to this problem, since it is high in protein, not used directly as a human food and it can be grown on food waste. A few studies have shown that feeding high amounts of yeast can reduce fish growth, but we don't yet understand why.

Yeast used as a protein supplement in aquaculture feed comes with a variety of pros and cons. Yeast contains a large amount of DNA and RNA and humans who consume high amounts can develop kidney stones and gout. Fish are thought to digest larger amounts of DNA and RNA safely, but this ability has not been fully investigated. Moreover, climate change and the ensuing increase in water temperature may stress farmed fish, and feeding live yeast may have a probiotic effect that improves stress and immune responses. More research and understanding may address these drawbacks and increase the replacement of fishmeal with yeast in order to improve the sustainability of fish farming.

A series of experiments were performed at the Swedish University of Agricultural Sciences (SLU) to determine negative effects of feeding high amounts of yeast to rainbow trout, a commonly farmed freshwater fish. First, we looked at the influence of replacing 60% of fishmeal with yeast on blood physiology and found that fish showed signs of impaired red blood cells, referred to as anaemia, possibly due to the high content of DNA and RNA in the yeast cells. We analysed the amount of amino acids, which make up protein

in the body, in the plasma and found that levels were similar between fish fed yeast and fishmeal, which indicated that protein uptake was not a problem. However, when 40% of fishmeal was replaced with live yeast, no signs of red blood cell anaemia were found, suggesting that levels lower than 40% replacement do not induce anaemia. Fish fed yeast had similar levels of cortisol, a stress hormone, indicating a normal stress response when netted out of the fish tank. It is beneficial that the fish were not further stressed by being fed yeast, but not ideal since the stress response did not improve.

In further studies, the impact of feeding different levels of inactivated and live yeast on gut bacteria and yeast (microbiota) was investigated to see whether these feeds disturbed normal gut microbiota and caused changes that may lead to reduced fish growth. Inactivated yeast did not considerably change the growth or make-up of bacteria and yeast in the gut, although feeding live yeast resulted in high amounts of gut yeast. Gut bacteria remained unchanged except when another yeast species, *Wickerhamomyces anomalus*, was fed. In another experiment, water temperature in the fish tank was increased from 11 to 18°C to determine whether this influenced the effects of feeding live yeast. We found that fish in warmer water had elevated cortisol levels and suppressed expression of inflammatory markers in the gut. The immune response was further suppressed when fish in warm water were fed live yeast, which may increase the risk of disease. This suggests that rainbow trout at 18°C were stressed, with suppressed immunity, and that feeding live yeast added further stress.

In conclusion, replacing more than 40% of fishmeal with yeast may reduce growth of rainbow trout by inducing red blood cell anaemia rather than deficiencies in amino acids or disturbed gut bacteria. In addition, feeding a different yeast species (*W. anomalus*) and feeding live yeast to rainbow trout in warm water should be avoided, since this disturbs fish gut bacteria and suppresses their immune response. In the future, the content of DNA and RNA in yeast should be reduced in order to enable inclusion of higher levels of yeast in fish feeds, while using inactivated yeast is recommended to avoid altered gut microbiota and immune responses, especially since fish will become more stressed with increased water temperatures due to climate change. Based on these recommendations and provided remaining problems are resolved in future studies, yeast has the potential to replace fishmeal in order to increase the sustainability of fish farming.

Populärvetenskaplig sammanfattning

Ökningen av världens befolkning har medfört en ökad oro över tillgången på livsmedel och risken för livsmedelsbrist under de under de närmaste åren. Vattenbruk, odling av fisk och vattenväxter, kan vara en möjlig lösning för att hantera en eventuell livsmedelsbrist, eftersom klimatförändringar och överfiske begränsar tillgången på vildfångad fisk som livsmedel. Inom akvakulturen används fiskmjöl, som gjort på vildfångad fisk, som en proteinkälla i foder till exempelvis lax och öring. Användandet av fiskmjöl har kritiserats som en icke hållbar produktion eftersom det ökar trycket på vilda fiskbestånd som kan användas som mat till människor direkt. Jäst (*Saccharomyces cerevisiae*), som ofta används till bakning och tillverkning av öl, kan vara en lösning på detta problem eftersom jäst har en hög proteinhalt, används inte direkt som mat till människor och den kan odlas på restprodukter som exempelvis matavfall. Några studier har visat att utfodring av höga mängder jäst kan minska tillväxten hos fisk, men vi har inte hela bilden klar för oss varför det är på det viset.

Användandet av jäst som ett proteintillskott i fiskfoder medför både för- och nackdelar. Jäst innehåller en stor mängd DNA och RNA och människor som konsumerar höga mängder kan utveckla njursten och gikt vid för hög konsumtion. Fisk kan däremot hantera större mängder DNA och RNA, men denna förmåga har inte undersökts fullständigt och mer forskning behövs för att säkerställa vilka nivåer som inte är skadliga för fisken. Klimatförändringar och den därmed ökade vattentemperaturen kommer med all säkerhet påverka fisken och därmed också hur vi skall utfodra odlad fisk. Genom att utfodra fisk med levande jäst i fodret kan en probiotiskeffekt erhållas som därmed förbättra både stresstolerans och immunförsvar hos fisken. Mer forskning måste till för att optimera utnyttjandet av jäst och andra fodermedel för att kunna minska på fiskmjölsanvändningen och därmed förbättra hållbarheten inom fisk och skaldjursodlingen.

En serie experiment utfördes vid Sveriges lantbruksuniversitet (SLU) för att undersöka möjligheterna i att använda jäst i foder till regnbåge, en vanligen odlad sötvattenfisk. Inga negativa effekter upptäcktes när fiskmjöl ersattes med upp till 40% av jästprotein i fodret istället för fiskmjöl. När aminosyrakoncentrationen i blodplasma analyserades, hittade vi inga större skillnader i absorptionsmönster mellan fisk som utfodrats med fiskmjöl- eller jästbaserat foder, vilket indikerade att upptagningen av protein inte skiljer sig åt mellan dieterna. När fiskarna utfodrades med högre halter (60% jäst) fann vi däremot tecken på anemi, att antalet röda blodkroppar minskade, troligtvis på grund av det höga innehållet av DNA och RNA i jästcellerna. Men när 40% fiskmjöl ersattes med levande jäst påvisades inga tecken på anemi. Fisk som utfodrats med jäst hade liknande nivåer av kortisol, ett stresshormon, vilket indikerar en normal stressrespons när de håvades upp från fisktanken. Det är viktigt att veta att fisk inte stressades ytterligare genom att bli matad jäst, men inte idealisk eftersom stressresponsen inte förbättrades, vilket vi hoppades på. I ytterligare studier undersöktes effekten av att utfodra olika nivåer av inaktiverad och levande jäst på bakterier och jäst i tarmen (mikrobiota) för att se om dessa foder störd den normala tarmmikrofloran vilket i sin tur kan leda till minskad fisktillväxt. Inaktiverad jäst förändrade inte väsentligt tillväxten eller sammansättningen av bakterier och jäst i tarmen, även om utfodring av levande jäst medförde högre mängder av jäst i tarmen. Sammansättningen av bakterierna i tarmen ändrades inte förutom när en annan jästart, *Wickhamomyces anomalus*, användes i fodret. I ytterligare ett försök ökades vattentemperaturen från 11 till 18°C för att bestämma om detta påverkade utfodringen av levande jäst. Fisk som levt i varmare vatten hade ökade kortisolnivåer och nedreglerat genuttryck av inflammatoriska markörer i tarmen. Gener för reglering av immunsvaret nedreglerades ytterligare när fisk i varmt vatten utfodrades med levande jäst, vilket kan öka risken för sjukdom. Detta tyder på att regnbåge som odlas vid 18°C stressades v med nedsatt immunitet som följd och att utfodring av levande jäst medförde en ökning av problemet.

Sammanfattningsvis kan en ersättning med mer än 40% jäst istället för fiskmjöl minska tillväxten hos regnbåge genom att framkalla anemi snarare än en brist på aminosyror eller en störd tarmmikroflora. Dessutom bör utfodring med andra jästsorter (*W. anomalus)* och användande av levande jäst i fodret till regnbåge i varmt vatten (undvikas eftersom det medför en störd tammikroflora och ett nedsatt immunsvar. I framtiden bör innehållet av DNA och RNA i fiskfoder minskas för att kunna öka inblandningsnivån av jäst i fiskfoder medan användandet av inaktiverad jäst rekomenderas istället för istället för levande jäst för att undvika en förändrad tarmmikrobiota och nedsatt

immunsvar, särskilt eftersom fisken blir mer stressad med ökade vattentemperatur beroende på ökade klimatförändringar. Utifrån dessa rekommendationer och fortsatta studier har jäst en god potential att kunna ersätta fiskmjöl som proteinkälla och därmed bidra till en mer hållbar fiskodling.

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