Fatty acid composition in Arctic char (*Salvelinus alpinus*) fed with red yeast biomass

- A comparison between large and small specimens

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Agricultural Programme – Food Science
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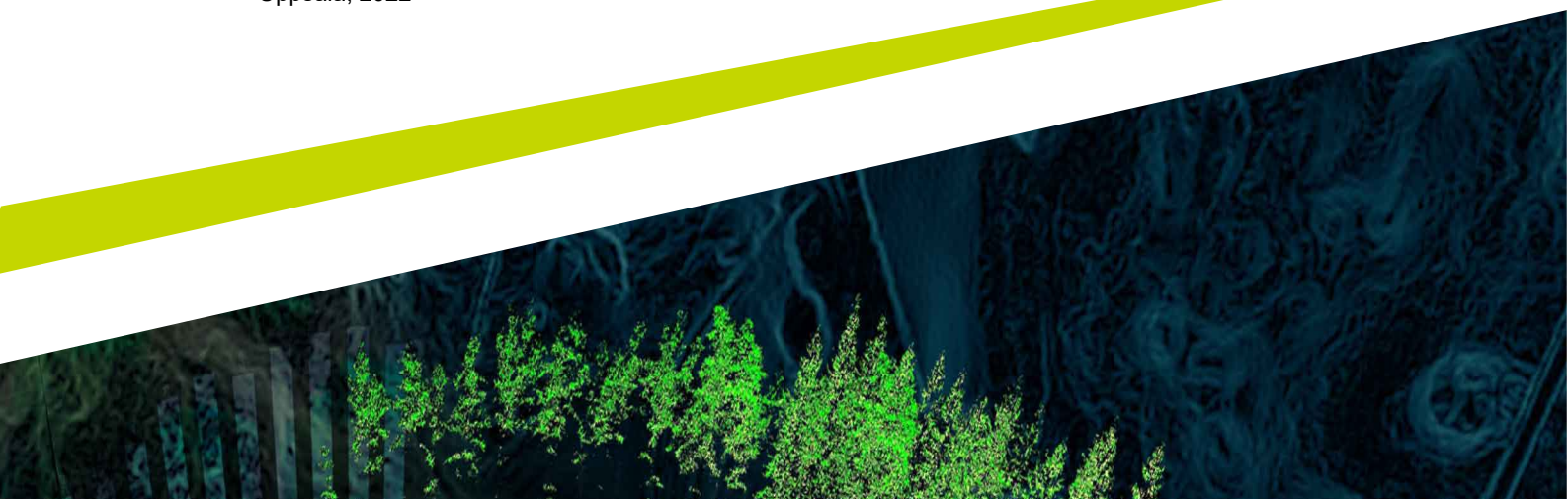

Fatty acid composition in Arctic char (*Salvelinus alpinus*) fed with red yeast biomass – a comparison between large and small specimens

Fettsyrasammansättningen i röding (Salvelinus alpinus) utfodrad med röd jästbiomassa – en jämförelse mellan stora och små exemplar

Sofia Jacobsson

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Abstract

Oleaginous yeast has shown to be a promising alternative to replace vegetable oils in fish feed. A problem in aquaculture is that different growth rates can be observed among farm-raised fish, supressing the productions full potential. When different growth rates are observed, it can be questioned if the fish assimilate the feed to the same extent. Therefore, the aim of this study was to analyse the lipid content, fatty acid composition and lipid groups of large and small specimens of Arctic char (*Salvelinus alpinus*), fed with either control feed or feed containing biomass of the oleaginous yeast species *Rhodotorula toruloides*, to examine the fish welfare, performance and intake of feed in fish of different size. Lipid extraction followed by gas chromatography was used to analyse the fat content and fatty acid composition in 16 fish. Furthermore, thin layer chromatography was performed to analyse lipids groups. No significant differences in lipid content, fatty acid composition or lipid groups could be observed between large and small specimens fed either control of yeast feed. However, small fish tend to have higher levels of polyunsaturated fatty acids. The result of this trial indicates that there is no difference is feed assimilation between large and small specimens of Arctic char (*S. alpinus*).

Keywords: fatty acid composition, lipid content, oleaginous yeast, Arctic char (*Salvelinus alpinus*), aquaculture

Sammanfattning

Lipidackumulerande jäst har visat sig vara ett lovande alternativ till att ersätta vegetabiliska oljor i fiskfoder. Ett problem inom akvakultur är storleksskillnader mellan fiskar som föds upp tillsammans, vilket hindrar produktionens fulla potential. Således kan tillgodogörandet av fodret hos fiskarna ifrågasättas när betydande storlekskillnader kan observeras. Därav var syftet med denna studie att analysera fetthalten, fettsyrasammansättningen och lipidgrupperna i stora och små exemplar av röding (*Salvelinus alpinus*), utfodrade med kontrollfoder eller foder som innehåller biomassa av den lipidackumulerande jästarten *Rhodotorula toruloides*, för att undersöka foderintaget hos fisk av olika storlek. Lipidextraktion följt av gaskromatografi användes för att analysera fetthalten och fettsyrasammansättningen, och tunnskiktskromatografi användes för att analysera lipidgrupper i 16 fiskar. Inga signifikanta skillnader kunde observeras gällande fetthalt, fettsyrasammansättning eller lipidgrupper mellan stora och små exemplar som utfodrade med kontroll eller jästfoder. Dock tenderade små fiskar att ha högre halter av fleromättade fettsyror. Resultatet i detta försök indikerar att foderassimilering inte skiljer sig åt mellan stora och små exemplar av röding (*S. alpinus*).

Keywords: fettyrasammansättning, fetthalt, lipidackumulerande jäst, röding (*Salvelinus alpinus*), akvakultur

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Abbreviations

FA	Fatty acid
FO	Fish oil
MUFA	Monounsaturated fatty acid
TAG	Triacylglycerol
TFA	Total fatty acids
SCO	Single cell oil
PL	Phospholipids
PUFA	Polyunsaturated fatty acid
LC-PUFA	Long chain polyunsaturated fatty acid
VO	Vegetable oil

1. Introduction

The global population is continuously growing and the demand for food is increasing. Increased use of natural resources combined with technological developments have tripled the agricultural productivity the last 60 years. Nevertheless, hunger and malnutrition are still major challenges around the globe (FAO 2017). Many of today's existing food production systems are facing challenges regarding long term sustainability. To fight the obstacles with malnutrition and hunger and at the same time utilize our natural resources in a sustainable way, new solutions need to be implemented in our food systems, including aquaculture production.

The total fish production has increased over the last decades and in 2018, FAO estimated a total production of 179 million tonnes, of which 82 million tonnes was produced by aquaculture. Around 10 % of the total production is used to produce fish oil and fishmeal, instead of being used for direct human consumption. Fish is an important food source in many countries and 17 percent of the global population´s intake of animal protein is provided by fish consumption (FAO 2020).

Fish oil and fishmeal are traditionally used in feed for aquaculture production, to provide the fish with essential amino acids and long chain fatty acids. However, the amount of fish oil used in feed has declined due to changes in supply and price, which are affected by increased demand from pharmaceutical industries and natural climatic consequences, such as El Nino. For instance, between 1990 to 2013, the amount of fish oil used in feed for Atlantic salmon (*Salmo salar* L.), dropped from 24 % to 11 % (Sprague et al. 2016). Despite the decrease of fish oil used in fish feed, aquaculture production is still an expanding production system, which result in an increasing demand for raw fish material used for feed production. To create a sustainable aquaculture production system, the industry cannot rely on wild fish biomass often derived from overexploited fish stocks.

Therefore, ongoing research is evaluating the possibility to replace fishmeal and fish oil in feed used for aquaculture. Terrestrial oils have been successfully included in aquafeed without affecting the growth of the fish (Bell et al. 2002, 2003; Pettersson et al. 2009). Nevertheless, vegetable oils (VO) can be used for direct

human consumption and require arable land to be produced. Therefore, alternative oil sources have been discussed lately, to replace vegetable oils in fish feed, where single cell oils derived from yeast, bacteria and algae has been suggested. Recent studies have shown that lipid accumulating yeast strains can be cultivated on lignocellulosic material, such as wheat straw hydrolysate (Brandenburg et al. 2018, 2021). For example, yeast biomass from *Lipomyces starkeyi*, grown on wheat straw hydrolysate, has shown to be an alternative to VO in fish feed used for Arctic char (*S. alpinus*), without affecting the growth of the fish. By including the whole yeast biomass in the fish feed, not only the VO could be replaced but also one of the protein sources (casein) (Blomqvist et al. 2018). This enables an oil production system that does not compete with human consumption and does not require arable land.

The growth of farmed fish depends on several factors in the aquaculture production system. Fish species, stock density, feed composition and feeding strategy are some factors influencing the growth and welfare of the fish. Arctic char (*Salvelinus alpinus),* a popular salmonid fish used for aquaculture in the northern countries, has shown to vary in size in aquaculture production, due to aggressive behaviour (Jobling et al. 1993). Aggressive behaviour supresses the availability of feed for the smaller fish, and it could be questioned if they assimilate the feed in the same extent as the larger fish. Therefore, the aim of this study is to analyze the lipid content, fatty acid composition and lipid groups in muscle tissue of large and small specimens of Arctic char (*S. alpinus*)*,* fed with either control feed or feed containing the lipid accumulating yeast species *Rhodotorula toruloides* grown in wheat straw hydrolysate*,* to examine the fish welfare, performance and intake of feed in fish of different size.

1.1 Background

1.1.1 Lipids

Lipids are a complex group of compounds which are essential in various biological functions, such as cell signalling, energy storage and acting as structural components of cell membranes. Lipids include, acylglycerols, sphingolipids, glycerophospholipids, phenolic lipids, sterols, lipoproteins, saccharolipids and polyketides, and are categorized based on functional and physiological properties (Fahy et al. 2011). Acylglycerols are esters consisting of glycerol and one, two or three fatty acids, forming mono-, di-, and triglycerides. Acylglycerols are neutral lipids, meaning that they are nonpolar. Triglycerides (TAG) are a major storage lipid and are mainly stored intracellular as lipid bodies. Membranes on the other hand, consists of amphiphilic lipids to isolate the cell from its surrounding by

creating a membrane bilayer. Common membrane lipids are glycerophospholipids, sphingolipids, fatty acyls, sterol lipids, saccharolipids and polyketides (Jones et al. 2019).

Fatty acids

Fatty acids are aliphatic carboxylic acids that are important components in triacylglycerols and phosphoglycerides. Fatty acids can be classified as either saturated or unsaturated depending on the structure of the carbon chain. In saturated fatty acids, all bond in the carbon chain is occupied with hydrogen, meaning that there is no double bond between carbon atoms. Unsaturated fatty acids can be further divided into monounsaturated and polyunsaturated fatty acids, depending on the number of double bonds present in the carbon chain. Monounsaturated fatty acids contain one double bond, whereas polyunsaturated fatty acids contain at least two double bonds (Frankel 2012).

Fatty acids consisting of 18 carbon or more in length, with at least 2 double bonds are defined as polyunsaturated fatty acids. They are divided into two major groups, n-6 and n-3, depending on the position of the first double bond in relation to the methyl end of the fatty acid (Leonard et al. 2004). If the fatty acids contain 20 or more carbons in length and at least 3 double bonds, they are classified as long chain polyunsaturated fatty acids (LC-PUFAs). Examples of LC-PUFAs are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Tocher 2015).

Plant lipids

About 350 crops species are used for lipid production, whereas palm kernel, soybean, rapeseed and sunflower seeds are the major sources for edible vegetable oils. Palm oil is mainly produced in Asia and accounts for 32 % of the market share of the total vegetable oils produced. Soybean oil production is located in Argentina, Brazil, China and the USA, and is the second largest source of vegetable oil. The third largest oilseed source is rapeseed, which is mainly produced in China, India, Canada, the USA and the European Union (Jones et al. 2019).

Plant oil mainly consist in the form of TAGs, with various fatty acid structures. More than 300 different FA have been identified in plants. Nevertheless, five FAs make up the largest proportion of the total FAs in plant oils; palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1n-9), linoleic acid (C18:2n-9) and α-linolenic acid (C18:3n-3) (Baud 2018).

1.1.2 Aquaculture

Capture fisheries and aquaculture is estimated to have reached a total production of 179 tonnes of fish in 2018, were aquaculture production contributed with 82 million tonnes. Aquaculture´s contribution to world fish production has increased from 25.7 percent in 2000 to 46.0 percent in 2018. Asia account for 88.7 percent of the total aquaculture production (Figure 1), with China as the major producer. A slowdown in aquaculture production has been observed in China the last decade, resulting in a reduced annual growth of aquaculture production globally. However, a high growth rate is still seen in several other major producers, such as Ecuador, Indonesia and Bangladesh (FAO 2020).

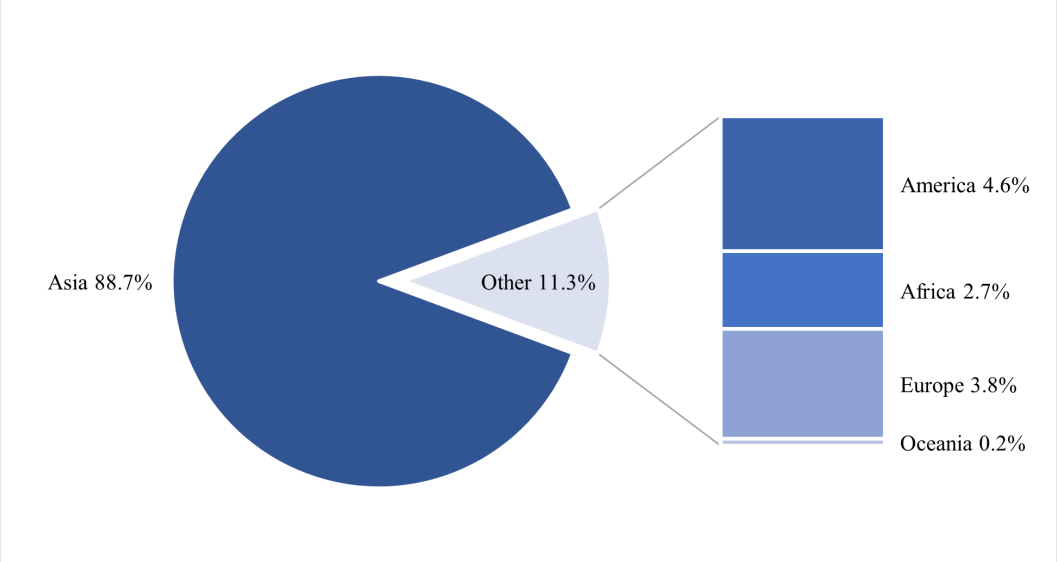

Figure 1. World aquaculture production by region in percentage, excluding aquatic plants and non-food products (source: FAO, 2018)

Most farmed aquatic animals are produced by inland aquaculture, which is mainly conducted in freshwater, using ponds, raceway tanks, pens and cages. Finfish is the main aquatic animal produced, accounting for almost 92 % of the total production. However, the production of crustaceans, such as shrimps, crabs and crayfish has been increasing lately, mainly in Asia. Coastal and marine aquaculture is performed in marine water environments, where coastal aquaculture includes production in gated lagoons and coastal ponds and marine aquaculture is based out in the sea (FAO 2020).

As the aquaculture production continues to expand, the demand for aquafeed is growing. A sustainable supply of fishmeal and fish oil is questioned due to limited wild-harvest resources. Therefore, plant-based material has been incorporated in

fish feed to substitute fishmeal and fish oil. However, these plant materials need to meet the nutritional requirement of farmed fish and be economically profitable, while contributing to a low environmental impact (Gatlin III et al. 2007).

Commercial fish feed is composed of various feedstuff such as fish meal, plant meals, wheat flour, rice flour, crushed corn, feather meal, bone meal, fish oil, vegetable oil, vitamins, minerals, and antioxidants. The composition and size of the pellets is designed to meet the nutritional requirement and growth stage of specific species. For example, protein and lipid requirements are higher for carnivorous species compared to omnivorous and herbivorous species (Boyd 2015). The inclusion of fish oil in the feed provides the fish with n-3 LC-PUFAs and the main LC-PUFAs found in fish are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which also are beneficial for human health due to its ability to prevent cardiovascular disease and hyperinsulinemia. All vertebrates, including cold-water marine species, such as Atlantic salmon (*Salmo salar*) lack the ability to produce PUFAs from oleic acid (C18:1n-9) and are therefore dependent on accessing these through diet (Tocher 2015).

Replacing fish oil and fish meal with plant materials

Plant oils are generally low in LC-PUFAs and contain high levels of saturated, monounsaturated and n-6 fatty acids compared to fish oil. Therefore, will VO not provide the fish with the essential n-3 LC-PUFAs (Torstensen et al. 2000). Studies show that fish oil can be successfully replaced with plant oils without affecting the growth of the fish (Torstensen et al. 2000; Bell et al. 2002, 2003; Pettersson et al. 2009). However, an alteration in fatty acid composition of the fish can be seen. A significant decrease of EPA (20:5n-3) was observed in Atlantic salmon (*Salmo salar)* and Arctic char (*S. aplinus*), when replacing 50 percent or more of the fish oil with plant oils (Bell et al. 2002, 2003; Fountoulaki et al. 2009; Pettersson et al. 2009). A significant reduction of DHA was observed in Atlantic salmon (*S. salar*) fed diets replacing at least 50 percent of the FO with rapeseed oil (Bell et al. 2003) or when replacing 100 percent of the FO with palm oil (Bell et al. 2002). However, no significant decrease in DHA could be observed in Arctic char (*S. alpinus*), between diets (0, 50, 75 or 100 percent rapeseed oil) in a study conducted by Pettersson et al (2009).

The fatty acid composition in fish muscle is greatly influenced by the diet of the fish. However, it is suggested that dietary fatty acids can be modified by the fish to maintain specific fatty acids levels in the muscle to support an optimal membrane function (Pettersson et al. 2009). Arctic char (*S. alpinus*) has shown to be able to reduce the n-6/n-3 ratio from 7.5 in the feed to 2 in the fish muscle, implying a mechanism to reduce n-6 PUFAs, that are of no structural importance in the fish

(Olsen & Henderson 1997). This reduction has also been seen in other salmonid fish (Hardy et al. 1987; Greene & Selivonchick 1990). It is also suggested that n-6 PUFAs are oxidized at a higher rate compared to n-3 PUFAs (Olsen & Henderson 1997).

In Europe, FO is commonly substituted with VO in aquafeed (Blomqvist et al. 2018). However, VO is widely used for biodiesel production, food production and direct food consumption. Therefore, it can be questioned if the inclusion of VO in aquafeed is sustainable from an environmental perspective. An increased demand for VO may encourage land conversion and rainforest cutting (Azócar et al. 2010).

Plant proteins are today the primary alternative protein source used in fish feed to replace fish meal. However, plant materials often contain antinutritional compounds which may affect the digestibility of the feed. Carnivorous fish, such as Salmonids, are more susceptible to the effect from bioactive compounds from plants and negative effects on digestibility has been observed in Atlantic salmon (*S. salar*) fed with soy bean meal (Krogdahl et al. 2003).

1.1.3 Oleaginous microorganisms

Microorganism accumulating lipids with more than 20 % of its biomass are defined as oleaginous. Oils produced by microorganisms such as algae, filamentous fungi and yeast are called single cell oils (SCOs) and are comparable with oils derived from plants and animals. Microbial cells accumulate lipids by converting carbon into storage fat, in the form of triacylglycerol. This is often caused by a limitation of a key nutrient, mostly nitrogen (Ratledge 2010). Substrates such as sugars, organic acids and carbon dioxide can be utilized for lipid production (Sitepu et al. 2014). The medium used for oleaginous species in lipid production need to contain a high C:N ratio to create a depletion of nitrogen while an excess of other nutrients, including carbon, is maintained. The optimal ratio may differ between individual species and growth conditions, nevertheless, a ratio of 30-80:1 is suggested (Jones et al. 2019).

Oleaginous yeast

Yeasts are unicellular fungi that have been utilized by humans for thousands of years, for instance in the production of wine, beer and bread, emphasizing the possibility of biotechnical application of yeast. Yeasts are easy to cultivate and there are only a few species known to be pathogenic to animals, plants or humans (Sitepu et al. 2014). Studies on high-oil producing yeast species, such as *Lipomyces starkeyi*, have been conducted as early as 1945 (Starkey 1945) and some of the most well studied oleaginous yeast species are *L. starkeyi*, *Rhodotorula toruloides*, *Cryptococcus albidus* and *Apiotrichum curvatum.* Oleaginous yeasts can utilize

various sources of carbon and accumulate lipids in the form of TAG and sterol esters (Jones et al. 2019). Yeast species such as *Rhodotorula toruloides*, have shown to be able to accumulate lipids up to 70 % of its biomass and the oil produced is comparable to conventional vegetable oil, as it consists of long-chain fatty acids (Li et al. 2007). Several studies demonstrate that some oleaginous species can utilize hemicellulosic and lignocellulosic material to produce TAG (Yu et al. 2011; Brandenburg et al. 2016, 2018), emphasizing the potential of an alternative TAG production with no extensive arable land requirement. Lignocellulosic material contains cellulose and hemicellulose, which can by pre-treatment and enzymatic hydrolysis, generate glucose, pentose and hexose sugars, which can be utilized as a carbons source by oleaginous yeast to produce lipids (Karlsson et al. 2016).

Advantages and limitations of SCOs

An advantage of SCOs compared to VO, is that it can be produced on land unsuitable for agriculture. Furthermore, microbial oils are not dependent on seasonal production and can be produced all year around (Sitepu et al. 2014). Since oleaginous microorganism can be cultivated on renewable resources such as lignocellulosic materials from agricultural residues, there is no competition with food production (Koutinas et al. 2014).

The major limitation of producing SCOs are the production costs, which include the fermentation process and post-processing of the lipids. The production is highly dependent on energy, meaning that the electricity price will greatly influence the total costs. Agitation and aeration of the bioreactors during fermentation accounts for the highest electricity consumption throughout the process and finding alternative agitation and aeration techniques would be needed to lower the energy costs (Karlsson et al. 2016). Post-processing of the lipids involves separation of lipids from the cell mass of the microorganism. The cell mass contains protein and carbohydrates and if the lipids are not separated from the microorganism, the whole microbial mass could be used as a source of lipids, proteins and carbohydrates. Calculations show that a price reduction of 17.6 % could be obtained if the lipids are not separated from the cell mass (Karamerou et al. 2021). Several studies has investigated the possibility incorporate yeast cells as a protein source in various fish species (Rumsey et al. 1990; Øverland et al. 2013; Vidakovic et al. 2016), where Arctic char (*S. alpinus*) (Vidakovic et al. 2016) and Lake trout (*Salvelinus namaycush*) (Rumsey et al. 1990) show no negative effect of replacing up to 40 and 50 % of the fish meal with yeast cells from *S. cerevisiae.* However, a lower final weight was observed in Atlantic salmon (*S. salar*) when replacing 40 % of the fish meal with yeast cells from *S. cerevisiae* (Øverland et al. 2013). By utilizing both the lipids and proteins in single cell organism, the separation during post processing of the cells can be avoided, and costs may be reduced.

1.1.4 Arctic char (*Salvelinus alpinus*)

Arctic char (*Salvelinus alpinus*) is a cold-water fish belonging to the Salmonidae family. It is distributed around the circumpolar north and populations are known to be riverine, lacustrine and anadromous (Wang et al. 2021). Their adaption to Nordic climate, makes the species optimal for farming in colder temperatures.

Farmed Arctic char show high growth rate during high stocking density compared with other species, such as Atlantic salmon. A hypothesis is that Arctic char possess more of a schooling behavior compared to Atlantic Salmon, meaning that high stocking densities may suppress aggressive behavior, resulting in higher growth rate of the fish. This is a positive feature when calculating production costs and capital investments for the production of farmed Arctic char (Wallace et al. 1988). However, this aggressive behavior in combination with social interaction of the fish has shown to result in size variation within rearing groups. Large variations in size can cause problems regarding harvesting strategy and food supply in commercial farming, preventing the production to reach its full potential (Jobling et al. 1993). Aggressive behavior can be caused by competition for space. The defended area (i.e territories) is influenced by several factors, such as food availability, body size and population density. Studies shows that high food availability typically decreases the territory size, whereas increased body size normally increases the territory size (Keeley & Grant 1995; Keeley 2000; Gunnarsson & Steingrímsson 2011).

In addition to availability of food, the feeding frequency also play a role in the growth of the fish. Arctic char (*S. alpinus*) has shown to grow better if they are fed more frequently compared to a few meals per day. Rainbow trout (*Oncorhynchus mykiss*), on the other hand, showed a reduced growth rate when fed frequently, which implies that different feeding methods are needed for specific species. Arctic char (*S. alpinus*) tend to be more resistant to stress and a more frequent feeding system increase the accessibility of food for each individual fish (Linnér & Brännäs 2001).

2. Materials and methods

2.1 Fish samples

The fish feeding experiment was performed by the Department of Molecular Sciences (Swedish University of Agricultural Sciences) at Kälarne Aquaculture North in Sweden. The experiment received the approval of the Ethical Committee for Animal Experiments in Umeå, Sweden and was carried out in compliance with EU legislation (*i.e.*, Directive 2010/63/EU). The fish, Arctic char (*S. alpinus*) (n=126) was raised at Kälarne Aquaculture North in Sweden, in a flow-through system of freshwater (10 L/min). The fish were kept in 1 x 1 m water tanks with a water depth of 20 cm. Commercial feed was used as feed to all fish before the trial. The fish tanks were randomly assigned either control (vegetable oil + casein) or experimental diet (yeast biomass) for a feeding period of 53 days. The vegetable oil consisted of a mixture of rapeseed and palm oil, to create a comparable fatty acid composition to the yeast oil. Cultivation of yeasts, production of feed and feed composition was similar as described in Blomqvist *et al*. (2018), except *Rhodotorula toruloides* was used as yeast strain in this trial. Fish were anaesthetised using tricaine methansulfonate and stored in -80 °C or -20 °C from 9 Sep 2019.

16 fish were chosen based on weight, length and feed, and thereafter divided into four groups; Large fish fed yeast feed (LY), Small fish fed yeast feed (SY), Large fish fed control feed (LC) and Small fish fed control feed (SC). Four fish (one from each group) was stored as whole fish in -20°C, and were filleted on the day of lipid extraction. 2 cm wide fillets were cut out transversally, starting from the end of the dorsal fin. Remaining fish muscle samples (n=12) was obtained as fillets stored in -80 °C until extraction.

2.2 Analyses

2.2.1 Lipid extraction

Lipid extraction was performed according to Pettersson *et al.* (2009) with slight modifications. Samples were partially thawed before skin was removed from the fillets. Samples were cut and minced with a knife, and all samples were extracted in duplicates. Approximately 1 g of sample were collected in Duran tubes for extraction and 8 ml Hexane: Iso-Propanol (HIP) (3:2; v/v) were added. The samples were homogenised for 3 x 30 s, using an Ultra Turrax (Janke and Kunkel, IKA, Werke, Staufen, Germany) and transferred to Teflon tubes. 6 ml of Na_2SO_4 solution (6,67%) were added and the tubes were vortexed. The samples were centrifuged at 4000 rpm for 5 min (Sorvall Super T21, Sorvall Products L.P., Newton, Connecticut, USA) and the upper phase of the samples were transferred into pre-weighed tubes. 2 ml of hexane were added to the samples in Teflon tubes which were centrifuged, and the upper phase was collected. This step was repeated twice. The samples were evaporated until dry, using N_2 and tubes were weighed. Lipids were dissolved in 0.5 ml hexane and stored in -20°C until further analysis.

2.2.2 Methylation

Methylation of lipids was performed according to Appelqvist (1968) with slight modifications, using BF_3 as reagent. Lipid samples were prepared by dissolving lipids in approximately 0.5 ml hexane to reach a concentration of 5 mg of lipids. Dry methanol was prepared with 0.01 M NaOH and 2 ml was added to each sample. Dissolved samples were heated for 10 min at 60 °C and 3 ml of BF_3 reagent were added to the tubes. The samples were vortexed and heated at 60 °C for a further 10 min. Thereafter, the samples were cooled down using ice bath. To each sample, 2 ml of NaCl (20 %) and 2 ml of hexane were added. The samples were vortexed and kept in 4°C for separation for 30 min. The upper hexane layer was transferred to a second glass tube, using a Pasteur pipette. The extraction was repeated by adding 1 ml of hexane, and the upper hexane layer was transferred, after 30 min of separation in 4 °C, into the second glass tube. Samples were evaporated under N_2 gas and thereafter dissolved in 0.5 ml hexane. The dissolved FAMEs were transferred to vials and kept in -20°C until further analysis.

2.2.3 Gas chromatography

FA methylation was checked by thin layer chromatography (TLC) using hexane, diethyl ether and acetic acid as solvent (85:15:2; v/v/v). 5 µl of each sample and standard (18:4) was added to a silica plate which was then placed in a TLC chamber for 1 hour.

FAME were analysed by using a gas chromatograph (CP3800, Varian AB, Stockholm, Sweden) equipped with a flame ionisation detector and a BPX 70 column (SGE, Austin Texas) (50 mm length, id 0.22 mm and a film thickness of 0.25 µm). FAs were identified by comparing their retention times with those of the standard mixture GLC 68A (Nu-check Prep, Elysian, USA).

2.2.4 Lipid class analysis

Lipid class analysis was performed according to Olsen and Henderson (1989) with slight modifications. Lipid samples were diluted with hexane to a concentration of 1 µg/µl. 5 µl of each sample was applied on a TLC plate (20x10 cm; Silicagel 60; 0.20 mm layer, Merck, Darmstadt, Germany) in 2 mm bands, 2 cm from the edge of the plate with a CAMAG TLC Sampler ATS4 (Camag, Switzerland). The application speed was 250 nl/sec and nitrogen gas were used as spray gas 9.8 mm distance was used between tracks and all samples were applied in duplicates. CAMAC Automatic Developing Chamber 2 (ADC 29) (Camag, Switzerland) was used for separation of lipid classes, using a mobile phase of hexane; diethyl ether; acetic acid (85:15:2; v/v/v). Thereafter, plates were dipped in a solution of cupric acetate (3%) and phosphoric acid (8%) and heated at 110°C for 20 min. Separated lipids were quantitatively analysed by scanning the plates with a CAMAG TLC Scanner 3 (Camag, Switzerland) at a speed of 20 nm/sec and a data resolution of 100 µm/step at a wavelength of 350 nm. External standards (TLC 18:4; Nu-Chek Prep, Elysian, USA) (TLC 18:5, Nu-Chek Prep, Elysian, USA) were used to identify lipid classes of the samples. The mode Savatisky-Golay 7 was used for data filtering, and peaks and baselines were corrected manually.

2.3 Statistics and calculations

Mean values and FA percentages of technical duplicates were calculated in Excel. Nonparametric Kruskal-Wallis tests followed by a pair-wise comparison (Dwass, Steel, Critchlow-Fligner multiple comparison post hoc procedure) in SAS was used to test the significant differences in weight, length, fatty acid composition and lipid class composition. Data is presented as median (min, max). Differences were considered to be significant at $p<0.05$. Trends of differences are presented at $p<0.10$.

3. Results

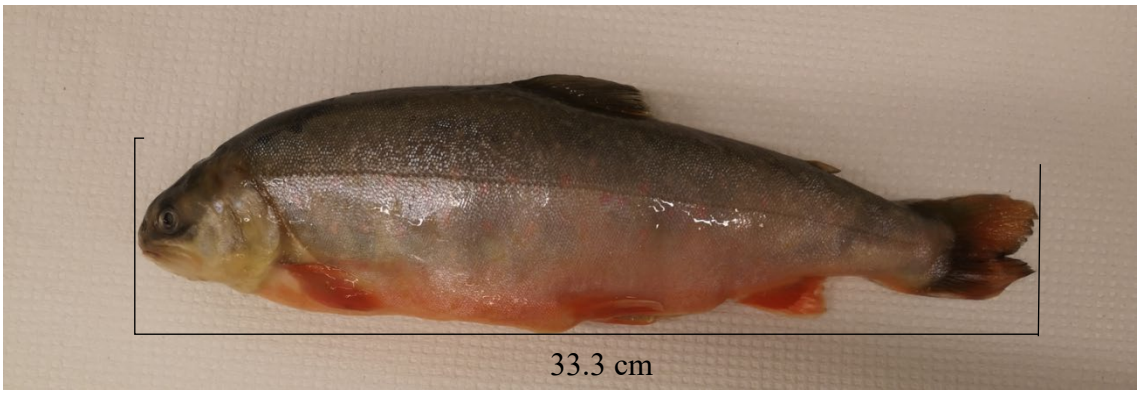

Figure 2. Large specimen of Arctic char (*S. alpinus*) fed yeast feed.

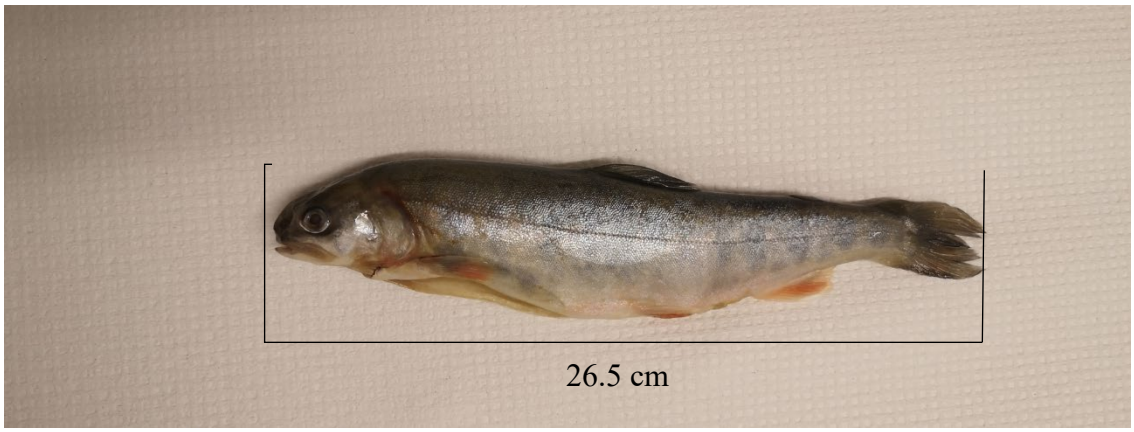

Figure 3. Small specimen of Arctic char (*S. alpinus*) fed yeast feed.

Figure 2 shows the large specimen of Artic char fed yeast feed that was stored at -20°C until lipid extraction, with a weight and length of 489.6 g and 33.3 cm. Figure 3 shows the small specimen of Artic char fed yeast feed that was stored at -20°C until lipid extraction, with a weight and length of 202 g and 26.5 cm.

3.1 Lipid content and fatty acid composition

The total weight, length, fat content and fatty acid profile of four large and four small control fed fish and four large and four small yeast fed fish are shown in Table 1. Presented p-value indicates overall effect of treatment according to a non-parametric Kruskal-Wallis test. Significant effect ($p<0.05$) of treatment was observed for palmitic acid (PA, C16:0), vaccenic acid (VA, C18:1n-7), linoleic acid (LA, C18:2n-6), docosahexaenoic acid (DHA, C22:6n-3), total PUFAs, n-3 and n-6. However, when pair-wise comparison was conducted with the Dwass, Steel, Critchlow-Fligner multiple comparison post-hoc procedure, no significant differences for these variables were observed between large and small fish fed with or without yeast. Yet, trends of differences could be observed ($p<0.1$).

Between control groups, large fish showed a trend of higher amount of palmitic acid compared to small fish. Nevertheless, small control fish tend to have higher amounts of LA (C18:2n-6) and DHA (C22:6n-3), compared to large control fish. Furthermore, a trend of differences could be observed in total PUFAs and total n-3 and n-6 in control fish, whereas small control fish tend to have higher content of PUFAs, n-3 and n-6. No similar trend could be observed between small and large fish fed yeast feed regarding n-3 and n-6 fatty acids, nevertheless a trend of differences could be observed in the total PUFAs, where small fish tend to have a higher level.

Table 1. Weight (g), length (cm), fat content (%) and fatty acid composition (% of total fatty acids, TFA) in Arctic char fed with either control or yeast feed (n=4 for each group).

	SY	LY	SC	LC	p-value
Weight (g)	179.2 # (167.4; 202.0)	346.5 ## (308.9; 489.6)	168.4 # (151.9; 211.7)	469.5 ## (410.4; 533.4)	0.007
Length (cm)	25.5 # (22.7; 26.5)	30.9 ## (28.0; 33.3)	25.1 # (24.7; 27.3)	32.5 ## (31.3; 33.0)	0.009
Lipids %	7.6 (6.3; 11.6)	9.4 (7.9; 10.4)	7.3 (3.5; 10.1)	13.1 (9.2; 16.6)	0.082
Fatty acid composition (% of TFA)					
C14:0	3.5 (3.4; 3.8)	3.6 (3.5; 3.6)	3.5 (3.1; 3.7)	3.6 (3.3; 3.8)	0.877
C16:0	14.0 # (13.5; 14.6)	14.2 # (13.9; 14.8)	14.1 # (13.9; 14.3)	15.1 ## (14.9; 15.5)	0.029
C16:1(n-9)	6.1 (5.4; 6.5)	6.2 (5.7; 7.0)	5.0 (4.3; 5.6)	5.8 (5.7; 6.4)	0.061
C18:0	2.0 (1.9; 2.3)	2.1 (1.9; 2.2)	2.1 (2.0; 2.2)	2.2 (1.2; 2.4)	0.830
C18:1(n-11)	1.2 (0.7; 1.3)	1.2 (1.1; 1.2)	1.3 (1.2; 1.3)	1.2 (1.1; 1.2)	0.083
C18:1(n-9)	28.4 (27.4; 28.5)	28.5 (27.9; 29.9)	27.5 (25.9; 28.6)	29.0 (28.6; 29.4)	0.069
C18:1(n-7)	2.8 # (2.8; 2.8)	2.7 # (2.6; 2.8)	2.8 (2.6; 3.0)	3.1 ## (2.9; 3.1)	0.044
C18:2(n-6)	6.9 # (6.5; 7.3)	6.5 (6.4; 7.0)	7.1 # (6.9; 7.2)	6.2 ## (6.0; 6.4)	0.024
C18:3(n-3)	1.8 (1.4; 2.2)	1.7 (1.7; 1.8)	2.0 (1.9; 2.1)	1.8 (1.7; 1.8)	0.068
C20:1(n-11)	1.1 (1.1; 1.1)	1.2 (1.1; 2.3)	1.1 (1.0; 1.1)	1.1 (1.0; 1.1)	0.337
C20:1(n-9)	6.8 (6.7; 7.4)	7.0 (6.9; 7.9)	6.8 (6.6; 7.1)	7.3 (6.9; 7.6)	0.201
C20:2(n-6)	0.5 (0.4; 0.5)	0.4 (0.4; 0.5)	0.5 (0.4; 0.5)	0.5 (0.4; 0.5)	0.613
C20:4(n-6)	0.4 (0.2; 0.6)	0.4 (0.4; 0.5)	0.4 (0.2; 0.5)	0.3 (0.0; 0.5)	0.576
C22:1(n-11)	7.2 (7.0; 7.3)	6.8 (6.6; 7.4)	7.2 (7.1; 7.5)	7.2 (7.0; 7.5)	0.368
C22:1(n-9)	0.8 (0.7; 0.9)	0.7 (0.7; 0.8)	0.8 (0.7; 0.9)	0.8 (0.7; 0.8)	0.485
C20:5(n-3)	3.8 (3.8; 4.0)	3.9 (2.1; 4.2)	4.0 (3.9; 4.3)	3.2 (3.1; 3.6)	0.053
C22:5(n-3)	1.0 (0.9; 1.1)	0.9 (0.9; 1.0)	0.9 (0.6; 1.0)	0.9 (0.9; 1.0)	0.326
C22:6(n-3)	10.3 # (9.5; 10.8)	9.4 # (8.8; 10.0)	11.7 # (9.6; 13.7)	8.8 ## (8.1; 9.3)	0.018
SFA	19.5 (19.0; 20.5)	19.8 (19.4; 20.6)	19.6 (19.4; 20.1)	20.7 (20.2; 21.1)	0.063
MUFA	54.2 (53.2; 54.5)	55.3 (53.4; 56.2)	52.4 (50.4; 54.5)	55.7 (54.1; 56.7)	0.075
PUFA	24.8 # (23.7; 25.2)	23.1 ## (22.3; 23.7)	26.8 # (24.0; 28.5)	21.8 ## (20.9; 22.0)	0.004
n-3	16.9 (16.1; 17.5)	15.5 (14.9; 16.3)	18.8 # (16.4; 20.3)	14.9 ## (13.9; 15.1)	0.007
n-6	7.8 # (7.6; 7.9)	7.4 # (7.2; 8.0)	8.0 # (7.6; 8.2)	6.9 ## (6.7; 7.2)	0.014
n-3/n-6	2.2 (2.1; 2.3)	2.1 (1.9; 2.2)	2.3 (2.2; 2.5)	2.1 (2.0; 2.3)	0.244

SY, Small fish fed with yeast feed; LY, large fish fed with yeast feed; SC, Small fish fed with control feed; LC, Large fish fed with control feed. Values are expressed as median (min, max).
Different numbers of #; ## indicate trends of differences at $p<0.10$

3.2 Lipid class analysis

Lipid class composition of phospholipids (PL), sterols, free fatty acids (FFAs) and triacylglycerols (TAG) expressed as % of total lipids in fish muscle samples are shown in table 2. Presented p-values indicate overall effect of treatment according to a non-parametric Kruskal-Wallis test. Significant effect ($p<0.05$) of treatment was observed for PL and sterols. However, when pair-wise comparison was conducted with the Dwass, Steel, Critchlow-Fligner multiple comparison post-hoc procedure, no significant differences for these variables were observed between large and small fish fed with or without yeast. Yet, trends of differences could be observed ($p<0.10$) (Table 2).

A trend of differences could be observed in phospholipids and sterols. Small control fish tend to have higher content of phospholipids compared to small fish fed with yeast fed. However, no trends of differences could be observed between large and small fish fed control or experimental feed. Furthermore, small fish fed control feed tend to have higher levels of sterols (5.3 %) compared to large fish fed control feed (3.3 %). TAG were the predominant lipid class (82.7 – 86.4 %), followed by PL (8.7-11.5 %) and sterols (3.3-5.3 %) in all four groups of fish (Table 2).

The maximum value of FFA was observed in fish (n=4) that has been stored in -20°C. These four fish also expressed the minimum value for TAG. Levels of FFA were not identified or identified at low levels in samples (n=12) stored at -80°C. Furthermore, samples stored at -80°C, express the highest values (maximum) of TAG. Peaks of an unidentified lipid group in the TLC lipid group analysis (Unknown, table 2) was observed in fish (n=4) that has been stored in -20°C. No peaks of the unknown lipid group could be detected in fish that has been stored in -80°C.

Table 2. Lipid class composition (%) in large and small Arctic char fed with either control or yeast feed (n=4 for each group.)

	SY	LY	SC	LC	p-value
Phospholipids	9.4 # (9.3; 10.3)	8.7 (7.2; 10.6)	11.5 ## (10.4; 12.9)	10.0 (9.5; 11.0)	0.027
Unknown	0 (0.0; 2.3)	0 (0.0; 2.2)	0 (0.0; 2.0)	0 (0.0; 2.0)	0.992
Sterols	4.6 # (4.0; 6.2)	4.0 # (3.9; 4.4)	5.3 # (4.7; 8.9)	3.3 ## (2.9; 3.7)	0.008
FFA	0 (0.0; 11.6)	0.1 (0.0; 7.3)	0.2 (0.0; 10.8)	0 (0.0; 6.1)	0.982
TAG	85.6 (70.7; 86.6)	86.0 (79.2; 87.7)	82.7 (66.3; 84.9)	86.4 (77.9; 87.6)	0.292

SY, Small fish fed with yeast feed; LY, large fish fed with yeast feed; SC, Small fish fed with control feed; LC, Large fish fed with control feed. Values are expressed as median (min, max).
Different numbers of #; ## indicate trends of differences at $p<0.10$

4. Discussion

The purpose of this study was to examine if any variations in fat content, fatty acid composition and lipid groups could be observed in large and small specimens of Arctic char (*S. alpinus*) fed either control feed or yeast biomass feed. The results indicate that there is no significant difference in assimilation of feed between large and small specimens of Arctic char (*S. alpinus)* fed either control or yeast feed. Replacing VO with oil produced from the yeast *L. starkeyi* has previously been tested in Artic char (*S. alpinus*), resulting in no significant difference in fatty acid composition, except for linoleic acid (C18:2n-6), where slightly higher levels could be observed in the fish fed with control feed (Blomqvist et al. 2018). This aligns with the results observed in this study, where no significant differences could be observed in fat content and fatty acid composition between fish fed with control or yeast feed, yet no difference of LA (C18:2n-6) could be observed between diets in the present study. However, small fish fed with control feed tend to have slightly higher levels of linoleic acid (C18:2n-6) compared to the large fish fed control feed. Oils derived from both plants and the oleaginous yeast *R. toruloides* contain linoleic acid (C18:2n-6) (Wu et al. 2021), which may explain why no significant difference was found between the diet groups.

Small fish tend to have higher levels of total n-3 compared to large fish in both control and yeast fed fish groups. n-3 fatty acids are mainly found in PL to maintain stability and functionality of cell membrane. When animals increase in fatness, the levels of PL stay fairly constant, or increase slightly. Neutral lipids, on the other hand, increase with the fatness of the animal and will constitute a larger part of the total fatty acid composition (Wood et al. 2008). Therefore, smaller animals would be expected to contain a higher proportion of n-3 fatty acid, found in the PL in the cell membranes. However, a significant higher content of PL could not be observed in small fish fed either control or yeast feed in this study.

Small fish tend to have slightly higher levels of n-6 fatty acids compared to large fish. Higher levels of both n-3 and n-6 fatty acids in small fish, results in a similar ratio of n-3/n-6 compared to large fish of both diet groups. Inclusion of VO in fish feed has shown to decrease the n-3/n-6 ratio in Artic char (*S. alpinus*) (Pettersson et al. 2009), which is not desirable from a health perspective. However, a decrease

in n-6 fatty acids has been observed in Arctic char (*S. alpinus*) when replacing VO and casein with yeast biomass from the species *L. starkeyi*, indicating that a desirable n-3/n-6 ratio could be obtained by replacing VO with yeast biomass (Blomqvist et al. 2018). A decrease in n-6 fatty acids in yeast fed fish could not be observed in the present study Nevertheless, the results indicate that replacing VO with yeast oil may not affect the n-3/n-6 ratio and that there is no significant difference between small and large specimens. To provide farmed fish with LC-PUFAs, which are lacking in VO, an alternative would be to use an oleaginous microorganism which can produce LC-PUFAs, such as the marine alga *Crypthecodinium cohnii* (Ratledge et al. 2001).

Trends of difference in fatty acid composition was more pronounced between large and small fish fed control feed. When observing the weight differences between all groups, there is a greater difference in weight between large and small fish fed control fed compared to large and small fish fed yeast feed. This implies that there could be a variation in fatty acid composition depending on size, thus the size difference in the yeast fed fish was not big enough to result in any significant differences.

No significant difference could be observed in lipid content between large and small fish in either diet groups. However, a large biological variation was seen in the four experimental groups. Biological variation has been seen in Atlantic salmon, when analyzing a homogenous group of 145 farmed salmon (Refsgaard et al. 1998). Variation in lipid content was found between fish when analyzing the same body part of the fish. Variation in lipid content between body parts was also observed, exemplifying the importance of body part used for extraction when analyzing fat content in fish. In the present study, one fish from each group (n=4) was filleted on the same day as the extraction, whereas remaining fish (n=12) was obtained as fillets and not whole fish. The large variation in lipid content could therefore be due to natural biological variation between fish, in combination with variation due to handling of the muscle tissue when filleting. This underlines the necessity to analyze a large number of fish to obtain a representative average of a population.

A storage effect could be observed in the fish that has been stored in -20°C, were high levels of FFA and lower levels of TAG was identified compared to fish which was stored in -80 °C. FFA are an indicator of lipid hydrolysis, which is caused by enzymatic activity. Enzyme activity is inhibited by frozen storage, nevertheless the storage temperature determines the efficiency of enzyme inhibition. Atlantic mackerel (*Scomber scombrus*) stored at -18°C has shown to be more susceptible to lipid deterioration compared to fish samples stored at -25°C (Romotowska et al. 2016) regarding FFA formation. This could explain the levels of FFA found in fish

stored in -20°C, where TAGs may have been degraded due to a higher enzymatic activity compared to -80°C. Furthermore, an unknown lipid group was identified in fish stored at -20°C, which was not detected in fish stored at -80°C. This may also be a degradation residue due to lipid hydrolysis.

The biggest limitation of producing SCO is the production cost (Karamerou et al. 2021). In this study, the whole yeast cell, including protein and lipids were used in the fish feed instead of vegetable oil and casein, which means that the extraction of lipids from the yeast cell is not needed. According to Karamerou *et al.* (2021), eliminating the extraction step can reduce the production costs up to 17.6 %. Further reductions in production costs can be achieved by identifying yeast species who possesses the ability to grow fast and accumulate lipids at high levels and by utilizing cheap material as substrate for the yeast (Karlsson et al. 2016). In this study, the yeast was cultivated on lignocellulosic material which is found in abundance from agricultural and forestry waste. Utilizing these materials may add value to waste streams of agriculture and forestry production and create a more sustainable production system.

The stocking density of the farmed fish in this study may not have been as high as the stocking density used in large-scale production of Arctic char (*S. alpinus*) due to the scope of the study. Small groups of fish can result in territorial behaviour and dominance hierarchies (Linnér & Brännäs 2001) which could be the reason why size differences were observed among the fish in this trial. A higher stocking density promotes schooling behaviour among fish, resulting in less dominance hierarchies. A higher stocking density could therefore reduce size variations in fish stocks. Nevertheless, Arctic char (*S. alpinus*) is by nature an aggressive species (Jobling et al. 1993), making it important to consider stocking densities, but also feeding strategies when creating an efficient aquaculture production.

The reason no significant difference was seen between groups in this study could be due to the low number of samples in each group. Because there were only four fish in each group, a normal distribution of the data could not be assumed and therefore the non-parametric Kruskal-Wallis method was chosen for statistical analysis. The statistical test shows an overall significant effect when comparing all groups, however, when performing a pairwise comparison, no significant differences could be observed, but only trends. To increase the reliability of the results, a larger number of fish should be analysed.

5. Conclusion

In summary, this study indicates no differences in assimilation of feed in large and small specimens of Arctic char (*S. alpinus*) fed control or yeast biomass feed. This implies a promising utilization of lignocellulosic material to produce SCOs, which could be able to replace VO in fish feed without affecting the growth of the fish.

5.1 Future perspectives

Since replacing FO with VO may affect the amount of LC-PUFAs present in the muscle tissue of fish, using different species or strains of oleaginous microorganisms, capable of producing LC-PUFAs would improve the fatty acid composition of SCOs used in fish feed. This would provide the fish with essential fatty acids and a larger part of the fish oil could be replaced with oils not dependent on overexploited fish stocks. This would also preserve the levels of LC-PUFAs available in fish that are important for human health. Future research on large scale cultivation of oleaginous microorganisms and downstream processing are required to create an efficient production of SCOs that can compete with today's production of vegetable oils.

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Popular science summary

Aquaculture is a continuous growing production system providing people with food rich in protein and healthy fats. Fish feed used in aquaculture production often contain both fish oil and fish meal to provide the farmed fish with essential nutrients. Fish oil and fish meal are mainly produced from wild fish stocks, and an increasing production of farmed fish will also increase the demand for fish oil and fish meal. Today, several wild fish stocks are fully exploited or overexploited, which means they cannot meet the demand for raw fish material used in fish feed, if the aquaculture production continue to grow. This has been addressed by replacing a part of the fish-based ingredients in fish feed with plant materials, such as vegetable oils. Vegetable oils are already widely used for human consumption and in food production and the ongoing shift from fossil-based fuels to more sustainable alternatives such as biodiesel, have extended the use for vegetable oils even further. Oil crops, as all plants, need arable land to be grown, and if the demand for vegetable oils would increase, this may lead to deforestation to be able to cultivate these crops to a larger extent. Therefore, it has been investigated if vegetable oil in fish feed could be replaced with oils produced from microorganisms.

A problem seen during farming of fish, is that size differences between fish can appear, which is believed to be due to aggressive behaviour of the fish. It can therefore be questioned whether fish of different size assimilate their feed to the same extent. Therefore, the aim of this study was to compare fat content, fatty acid composition and lipids groups in large and small fish of the species Arctic char fed a feed containing biomass from a lipid accumulating yeast strain. Lipids were extracted and analysed to determine fat content, fatty acid composition and lipid groups in 16 fish. Overall, no significant difference could be observed between large and small fish fed either yeast or control feed, comparing fat content, fatty acid composition and lipid groups. Thus, small fish tend to have higher levels of polyunsaturated fatty acids compared to large fish, which could be explained by a higher proportion of membrane lipids instead of stored fat in the form of triacylglycerols. However, higher levels of membrane lipids could not be observed in small fish in this study. Further studies should include a larger number of fish to

increase the accuracy of the experiment. This study was limited by the number of fish available for analysis. In summary, this study indicates a similar assimilation of feed between large and small fish fed both yeast and control feed with no significant differences in fat content, fatty acid composition or lipids groups.

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