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Alternative oils in cod diet. Effect on fish performance.

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Abstract

A study was conducted to investigate the effect of different sources of feed oils in the diet for cod. During the experiment we tested four types of feed. The content of protein (53%) and starch (8.5%) were the same in all four diets, while fat percentages were 19.1 % in FORO diet, 17.8 % in SO-L diet, 17.2 % in SO-H diet and 20.2 % in FO diet. The type of oils was different in all dietary groups.

Particular, focus was given to evaluate the different type of oils used; a rapeseed oil/fish oil blend (FORO), salmon oil of low quality (SO-L), salmon oil of high quality (SO-H) and normal fish oil (FO). We wanted to evaluate how the different oils affected the growth performance, the lipid and fatty acids composition of the cod liver and health parameters of the experimental fish.
In our study, we showed that none of the tested oils had negative effects on growth, feed efficiency and health parameters of the fish, in despite of higher tendencies to peroxidation processes in SO-H and SO-L groups.

The results of the present thesis suggest that salmon oil can be used as a good replacement for fish oil in the diets of cod.

Table of contents

1. Introduction

In recent years, marine culture of gadoids fish like cod has expanded, and this has increased the pressure on traditional feed sources like fish meal (FM) and fish oil (FO). One of the serious problems in maintaining growth in aquaculture is future provision with FO, used to feed carnivorous cultured species. It is expected that the use of FO will increase dramatically, and future expansion of aquaculture, in particular marine fish like cod, can continue only if alternatives to the traditional FOs are developed and introduced.

Many research projects have been done to investigate different alternative sources of feeds for farmed fish. By-product from fisheries and farmed fish has been tested for its adequacy as a substitute to FO. Accordingly, salmon by-product has been identified as a good candidate for addition or partly replacement of FO in diets for cod. Important topics to consider when evaluating salmon oil (SO) by-product sources are the effects on fish growth, health, welfare, and the final product quality.

1.1 Aim of the work

The aim of the present study was to evaluate the prospect of utilizing SO or rapeseed oil (RO) in cod diets, and further to evaluate how these oils affect the growth, lipid class and FA composition of the cod liver. Further, we wanted to evaluate how inclusion of RO and two different SO qualities affects oxidative stress parameters.

2. Background

2.1 Cod biology

Atlantic cod (*Gadus morhua*) is a cold-water species that prefer coastal water of the North Atlantic and can be found along coastal line of North America from Greenland to North Carolina.

Cod is a very adaptive species and can tolerate wide range of environmental variations. Adult cod is a bottom fish. Suitable temperature for cod is in range from 2 to 8°C (http://www.ucd.ie/codtrace/codbio.htm). However, during feed migration cod has been found in range up to 20°C. Young fish has special adaptive enzymes, working as anti –freezing enzymes and allow cod to tolerate very low temperature.

Adult cod are strictly carnivore fish. They have a wide spectrum of nutrition and can feed on any animals that they can swallow. It was suggested that cod has a special enzyme that enable them to digest chitin and give possibility for cod to consume animals with hard shells like crabs, shrimps and brittle stars (Morris & Green., 2002). Usually cod diets are rich in worms, mussels, squids, crustaceans and fishes such as sand eel, Norway pout, capelin, stick-backs, sprat and herring (http://www.ucd.ie/codtrace/codbio.htm). It is found many evidences that cod show cannibalistic behavior as well. It is suspected that type of diets may affect their skin color; cod that prefer to feed on crustaceans get a brownish-golden skin while cod that feed on fishes has more greenish-blue colors of the skin.

The wild cod has been an important marine resource for many years. In 1980s Norway started with commercial cod farming. The first breeding program was introduced in 2002. Today there are two commercial breeding programs for Atlantic cod in Norway.
Cod aquaculture is still a young industry, which have not been through many rounds of selection and domestication. This still result in large genetic variations among the fish.

2.2 Cod nutrition requirements

Cod is a marine, carnivorous species, and the natural diet for cod is rich in protein and fat. Correct balanced nutrition (protein, lipid, carbohydrates and vitamin) for the farmed fish is vital for good performance (Hemre et al., 1988; Lie et al., 1988; Morais et al., 2001; Grisdale-Hellad et al., 2008). As for other species, optimal diet for cod must always be based on knowledge.

An optimal diet for cod should be balanced with all essential nutrients and provide the fish with good growth, feed efficiency, high utilization and retention of nutrients, good product quality and at the same time be economical (Lie et al., 1988; Roselund et al., 2004; Grisdale-Hellad et al., 2008).

It was suggested that fish do not have exact protein requirement, but have a requirement for a well balanced mixture of essential and non essential amino acids (EAAs) that are important for good growth and health of farmed fish (Wilson, 2002; Webster & Lim, 2002). Any imbalance of composition in EAAs will evidently affect protein synthesis in the fish, growth rate, feed conversion and health of the fish (Wilson, 2002).

There are some important factors which can influence the protein requirement of farmed fish. Protein quality is one of the most important factors. Different protein sources have different content of EAA. Traditionally used FM provides fish with high quality protein which is rich in EAA, while some proteins of plant origin lack or have low level of some EAA (Hardy., 1996; Albrektsen et al., 2006). Secondly, an optimal balance of protein for cod depends on the dietary protein to energy balance. It was suggested that cod prefer to use protein as energy source, if the balance between dietary protein and lipid is sub-optimal (Morais et al., 2001; Wilson, 2002). Since FM is normally the most expensive nutrient in the cod diet, an aim has often been to supplement the cod diet with non-protein energy sources, which allow the fish to use the AA from the dietary protein more efficiently for protein synthesis and not for energy production, and at the same time give maximum growth for the cultured fish (Morais et al., 2001; Wilson, 2002; Webster & Lim, 2002). It was demonstrated better growth performance, improved protein efficiency and a protein sparing effect by changing the protein to lipid ratio from 58:12 to 48:16 (Morais et al., 2001). It has also been shown that small fish has higher requirement for protein

compared to larger fish. Finally, the last factor influencing the protein requirement is the water temperature. It is suggested that fish has higher protein request when temperature is optimal in range from 8 to10 °C (Bjorn Bjornsson et al., 2007).

The protein requirement for cod in order to obtain good growth and protein retention should contain 500-600 g raw protein/kg feed (Roselund et al., 2004; Einen et al., 2007; Grisdale-Helland et al., 2008).

The lipid has a very important role in the marine fish nutrition. The lipid requirements for cod can be divided into three main groups, gross energy provision, qualitative and quantitative essential fatty acid (EFA) requirements (Sargent et al., 2002).

Lipid is the main source of cheap and quality energy for cod. It was demonstrated that lipid is a more efficient and economical energy source in the diet, compared to proteins or carbohydrates (Sargent et al., 2002)

Different species have specific requirements for different FAs. Fresh water species can convert 18 carbons FAs, such as α-linolenic (LNA, 18:3 n-3) to 20 and 22 carbons FAs such as EPA and DHA, and linolenic acid (LA, 18:2 n-3) to arachidonic acid (AA, 20:4 n-6) (Sargent et al., 2002). Therefore requirements of EFAs for Atlantic salmon can be met with sufficient amount of LNA and LA and addition mixture of EPA and DHA (Ruyter & Thomassen, 1999). In contrast marine fish like cod are unable to elongate and desaturete 18:3n-3 to the EFAs such as EPA and DHA (Sargent et al., 2002), possible due to non functional desaturase and elongase enzymes in the EFA metabolic pathways (Tocher et al., 2003b; Tocher et al., 2006; Zheng et al., 2009). Hence, the requirements of EFAs for these species can only be met by additional supply of EPA and DHA to the diet (Sargent., 1999, 2002). It was determined that supply of EFAs in the diet not only promotes good growth in fish but also provide fish with good nutritional properties which is essential for human nutrition (Mourente & Bell, 2006).

One of the most important challenges related to dietary lipid for farmed cod is unwanted excessive deposition of fat in the liver, which raises the liver index. It has been reported that lipid accumulation in the liver may be directly related to dietary lipid and intake (Lie et al., 1986;

Jobling et al., 1991; Kjær et al., 2008b; Hansen et al., 2008) or due to imbalanced protein to lipid ratio or feeding regime in the diet (Roselund et al., 2004).

It was recommended that feed for cod should contain 130-200 g fat/kg feed (Roselund et al., 2004; Einen et al., 2007; Grisdale- Helland et al., 2008), to obtain optimal growth rates without producing fatty liver.

Carbohydrates are necessary components of fish diets. However, carnivorous fish species like cod have a very low capacity to digest complex carbohydrates, even at low dietary level (Hemre et al., 1988, 1991). It has been usual to use some carbohydrate as cheap component and good stabilizer for feed pellet in the diet for farming cod (Hemre et al., 1991). Many studies were done to evaluate how carbohydrates affect the fish and how much carbohydrates the feed should contain. It was suggested that growth rate, digestibility and feed utilization was negatively correlated with dietary starch level (Hemre et al., 1991; Einen et al., 2007; Albrektsen et al., 2008; Grisdale-Helland et al., 2008). Recommended level of starch in cod diet should be in the range of 120-150 g/kg feed (Roselund et al., 2004; Einen et al., 2007).

It is crucial to optimize the cod diet with proteins, lipids and carbohydrates. Any deficiency or excess may be negatively correlated with growth or quality of the fish, the environment or the economy.

2.3 Lipids in cod nutrition

As mentioned above, lipids play a central role in fish nutrition, particularly as an energy source, and as structural components of membranes as bio-active molecules (Sargent et al., 2002). Three important classes of lipids are FAs, triglycerides (TAGs) and phospholipids (PLs).

Fatty acids

FAs are simple lipids, formed from a hydrophilic carboxylate group which is attached to a hydrocarbon chain (Mathews & Holde, 1990; Voet., & Voet., 1995). FAs are basic components for all lipids, and are very seldom found as free molecules. There are a few factors that

characterize FAs; the length of the carbon chain, the degree of un-saturation, and the location of the first double bond from the methyl end (Sargent et al., 2002).

Accordingly, FAs can be divided into two groups; saturated FAs which have single bonds and unsaturated FAs that have one or more double bounds (figure 1). FAs with one double bond are called monounsaturated FAs (MUFAs). FAs that have more than one double bond are called polyunsaturated FAs (PUFAs), while FAs referred to as highly unsaturated (HUFAs) have three or more double bonds (Mathews & Holde., 1990; Voet & Voet., 1995; Sargent et al., 2002).

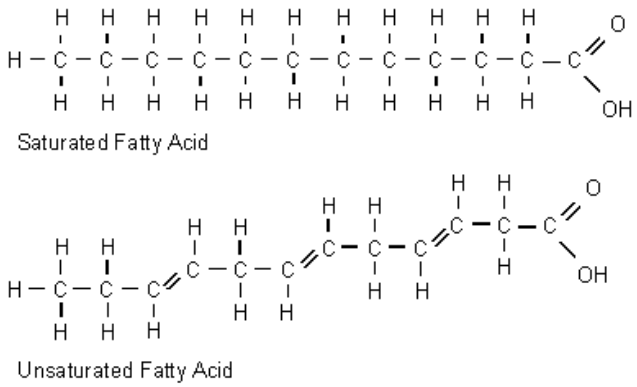

Figure 1 - General structure of saturated and unsaturated fatty acids. Figure was adapted from the net (http://faculty.clintoncc.suny.edu/faculty/michael.gregory/files/bio%20101/bio%20101%20lectures/biochemistry/biochemi.htm).

All these FA groups are important in fish nutrition with crucial roles as energy sources and in cell structure, and have further important roles in physiological functions of biological membranes (Sargent et al., 2002; Tocher, 2003a).

Triacylglycerols

TAG is an optimal storage form for energy in the animal. TAGs or neutral lipids are formed as triesters of FAs and glycerol, where R1, R2 and R3 position of the glycerol have different FAs attached (figure 2). TAG contain various FAs, but in most cases they have saturated FAs and MUFAs situated in the R1 and R3 position of the glycerol and PUFAs situated in the R2 position of the glycerol (Mathews & Holde., 1990; Voet & Voet., 1995).

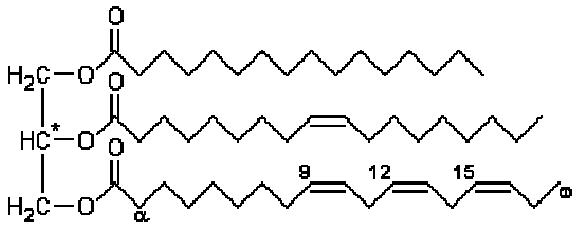

Figure 2 - Structure of triacylglycerol. Figure was adapted from the net (http://en.wikipedia.org/wiki/File:Fat_triglyceride_shorthand_formula.PNG)

The major function of TAG is to be an energy reservoir in the fish body, which is easy mobilized from cells when needed. Most of the TAG in fish is accumulated and stored as fat droplets in the cytoplasm in cells called adipocytes. Each fish species have a specific lipid storage place in the body. In cod, liver is the main lipids storage organ and sometimes account for 80% of total lipids in the body (Grisdale-Hellad et al., 2008; Hansen et al., 2008), while Atlantic salmon prefer to store lipids in the myosepta surrounding muscle fibers (Zhou et al., 1995) and in the visceral adipose tissue (Sheridan., 1994).

Phospholipids

PLs is another class of lipids that is essential as structural components of cell membranes. PLs have almost similar structure as TAGs, except that one of the FAs is substituted by a molecule containing phosphate. The R1 position of the glycerol is most often esterefied with saturated FAs or MUFAs, R2 position with PUFAs and a phosphoryl group is found at R3 position (Mathews & Holde, 1990; Voet., & Voet., 1995). Figure 3 shows the general structure of PLs. PLs differs according to phosphatidic acids. The most important PLs in the fish tissue are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI) (Tocher et al., 2008).

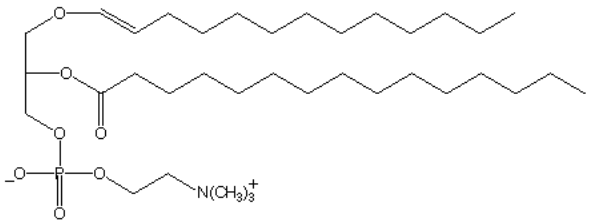

Figure 3 - General structures of phospholipids. Figure was adapted from the net (http://tonga.usip.edu/gmoyna/biochem341/lecture28.html)

Specific properties of PLs make them important structural components of cell membranes. They influence diverse functions such as permeability, fluidity and flexibility of the biological membrane (Sargent et al., 2002; Tocher et al., 2008).

2.4 Alternative oils in cod nutrition

FO replacement is not a new concept, and it has been given considerable attention in recent years, due to predicted increase of fish farming activity and the limited supply of FO on the world marked (Roselund et al., 2006). Fish feeds producers focus on supplementation of fish feed with alternative oil sources from plant and marine by-products. The use of alternative oils in the diet for cod should support similar fish performance with low negative effect for the fish and the environment (Tibbetts et al., 2006).

Vegetable oil (VO) is a good and widely available lipid source, and it is shown to be a successful substitute up to 50 to 70 % of FO for fresh water fish. However, VO has limitations as a sole lipid source for marine fish because marine fish like cod have low ability to elongate and desaturete 18:2n-6 and 18:3n-3, which are abundant in VO, into arachidonic acid (ARA), EPA and DHA (Sargent et al., 2002). It was suggested that partial replacement of FO would be possible if the requirements of EFAs were sufficient in the diet (Fountoulaki et al., 2009). Another problem related to VO is the alteration of the n-3 HUFA profile, in particularly the reduced level of EPA and DHA that is negatively reflected with quality characteristic on farmed fish. It is well known that the FA profile of the fish reflects the FA composition of the diet (Lie et al., 1986; Bell et al.,

2001, 2003; Jobling., 2008). Therefore, feeding diets high in VOs have the potential to reduce human health benefits associated with eating fish (Seierstad et al., 2005).
In contrast, by-products from fisheries and aquaculture seem to be a good source which may be considered as alternative FO sources in cod nutrition (Pickova & Mørkøre., 2007; Sørensen & Denstadli., 2008). One of the main advantages of by-product oils from aquaculture is that the ingredients of marine origin contain high levels of n-3 FAs compared to VO, especially HUFAs, and reflect the natural diets in the marine environment, which promote good fish health. However, processing of by-products it is still a costly process, which require knowledge and equipment.

2.5 Oxidative stress and peroxidation of lipids

The diets of cod are containing high amounts of PUFAs, especially the n-3 HUFAs EPA and DHA. The HUFAs, which are crucial constitutes for cell membrane structure and biological function in marine fish are highly susceptible to oxidative damage (Sargent et al., 2002). Oxidative damage to lipids or lipid peroxidation (LP) often happens in response to oxidative stress (OS) in the body or intake of rancid feed (Catala., 2008).

OS is defined as an imbalance between the prooxidant and antioxidant systems that predominate generation of reactive oxygen species (ROS) over the antioxidant defenses (Ott et al., 2007). ROS is a complex term that combines free radical and nonracial derivatives of oxygen, which are highly reactive due to the presence of unpaired electrons and can result in significant damage to cell structure (figure 4).The mitochondrial respiratory system is the major site of ROS production. At the same time, mitochondria are the most vulnerable target for oxidative damage by ROS (Ott et al., 2007).

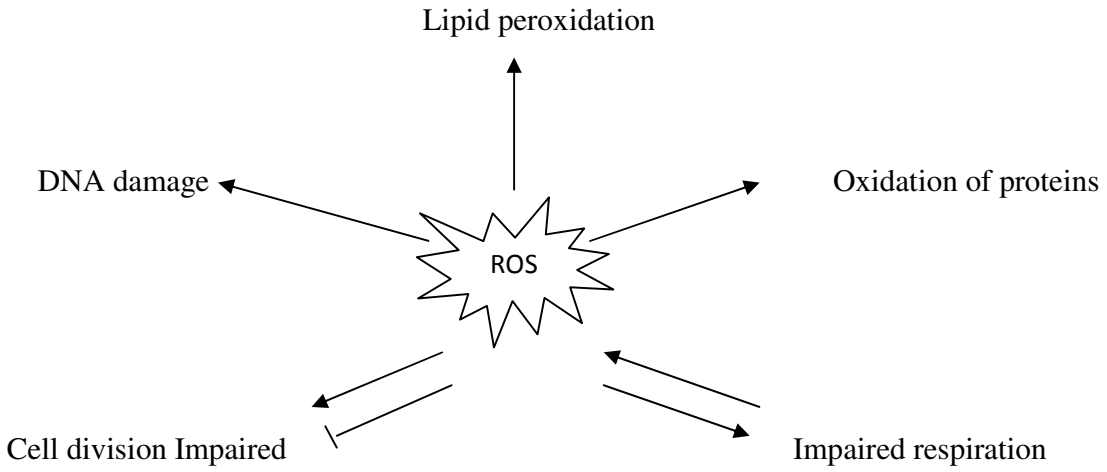

Figure 4 - Effects of reactive oxygen species on cellular functions.

LP is a free radical chain reaction, which is provoked by hydroxyl radicals (OH) and affects PUFAs, resulting in oxidative damage (Wiseman, 1996). LP has three main stages of development: initiation, propagation and termination (figure 5) (Catala, 2008).

The high susceptibility of PUFAs to peroxidation can be explained by the presence of multiple double bonds and methylene (-CH_2-) groups which contain highly reactive hydrogens.

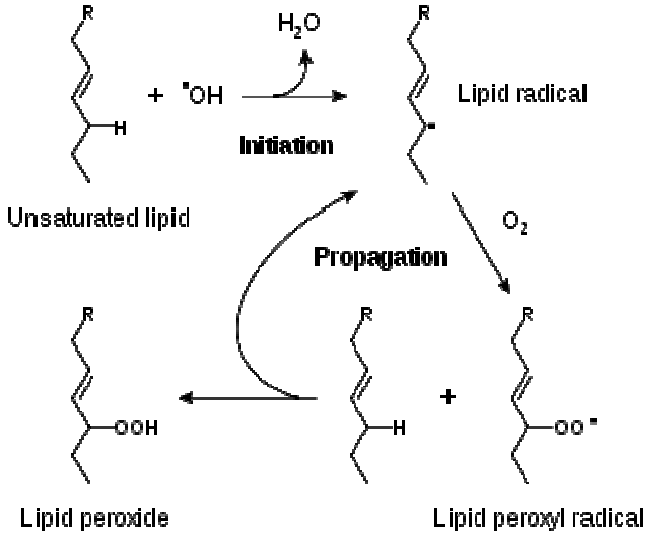

Figure 5 - Mechanism of lipid peroxidation. Figure was adapted from the net (http://en.wikipedia.org/wiki/Lipid_peroxidation).

The main products of peroxidation includes: 4-hydroxy-2-nonenal (HNE), 4-hydroxy-hexanal (HHE) and malondialdehyde (MDA). Production of these is indicating LP in vivo and in vitro (Halliwell & Gutteridge., 2007). Products of LP are highly reactive and may interfere with vital bio-molecules in the cell (Wiseman., 1996; Catala., 2008).

The consequence of LP can be sever, including damage to membrane function, causing changes in membrane fluidity and permeability, alteration of ion transport, inhibition of metabolic processes and in some cases apoptosis (Wiseman., 1996; Catala., 2008). Some resent studies show that high level of lipids and especially n-3 HUFAs in the diets for Atlantic salmon may result OS in the liver (Kjær et al., 2008a), adipose tissue (Todorcevic et al., 2009), and oxidative damage to muscle mitochondria (Østbye et al., 2009). To prevent oxidative damage, farmed fish should have an effective antioxidant defense system that partly should be covered with dietary supply (Martínez-Álvarez et al., 2005).

2.6 Antioxidant defense against oxidative stress and lipid peroxidation

An antioxidant has been defined as a molecule that is capable of delaying or preventing the oxidation process of a substrate. The fish has two main antioxidant defense systems, enzymatic and non-enzymatic. The enzymatic antioxidants are enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferase (GST) and glutathione (GSH) (Trenzado et al., 2006; Halliwell & Gutteridge, 2007). Non-enzymatic antioxidant includes vitamins, minerals and other molecules (Udilova et al., 2003; Martínez-Álvarez et al., 2005; Trenzado, 2007).

SOD is one of the most important enzymes, which metabolizes superoxide anion into molecular oxygen and hydrogen peroxide. CAT breaks down hydrogen peroxide to molecular oxygen and water. Hydrogen peroxide and organic hydroperoxides may be destroyed by GPx, with the presence of the tripeptide GSH. GST is a multifunctional enzyme, which is involved in the detoxification of xenobiotics and highly reactive electrophilic components (Sies, 1986; Halliwell & Gutteridge, 2007).

The fish antioxidant defense system is partly dependent on dietary provision with important antioxidants, such as vitamins E and C. Vitamin E (α-tocopherol) is the main lipid soluble antioxidant that is important in cell membranes protection from oxidation, especially with high HUFA levels in the diet of farmed fish (Udilova et al., 2003; Trenzado, 2007). Vitamin E is a chain-breaking antioxidant, which can scavenge free radicals such as lipid peroxyl radicals. This may explain the correlation between high level of lipids and vitamin E found in many studies (Udilova et al., 2003; Deaton & Marlin, 2004; Martínez-Álvarez et al., 2005). It has been reported that vitamin E level in both tissue and plasma were influenced by dietary lipid; the amount of vitamin E was decreased with high amount of n-3 HUFAs (Sargent et al., 2002; Puangkaew et al., 2005).

Vitamin C (ascorbic acid) is another non-enzymatic antioxidant, which act as a co-substrate in biosynthesis and can scavenge number of free and antioxidant-derived radicals. Vitamin C has important role in regeneration of vitamin E from radicals, and it was suggested that requirement of vitamin E depend not only on dietary lipids, but also on vitamin C. This interaction between vitamins C and E may be considered as a synergistic relationship in fish (Sargent et al., 2002). It was also reported that vitamin C play important role in plasma lipid peroxidation (Martínez-Álvarez et al., 2005; Puangkaew et al., 2005) and can improve blood oxidative status (Trenzado et al., 2009).

Carotenoids are a group of organic molecules, such as β-carotene, astaxanthin, and canthaxanthin which possess antioxidant properties, and have important correlation with vitamin E. Carotenoids are able to scavenge oxygen and peroxyl radicals (Sargent et al., 2002; Deaton & Marlin, 2004).

Studies in fish nutrition also have shown important role of some minerals such as manganese (Mn), copper (Cu), selenium (Se), and zinc (Zn) in protection from oxidative stress and lipid peroxidation (Martínez-Álvarez et al., 2005).

In addition, synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butylhydroquinone (TBHQ), propyl gallate and ethoxyquin are often used in the feeds in order to prevent the feed components from oxidative damage. In most cases, synthetic antioxidants are used when a longer shelf life is needed.

It has been considered that an antioxidant may protect one system, but may be useless to protect another one, or even in some cases can promote damaging effects on bio-molecules. Antioxidant inhibitors of lipid peroxidation may be inactive to protect molecules like DNA and protein against oxidative damage, and sometimes they contribute to oxidative damage of these molecules (Halliwell et al., 1995).

An antioxidant defense system is an important mechanism, which are essential for the fish. This system is responsible for maintaining the redox status of the cell and protects a fish from oxidative damage.

3. Materials and methods

3.1 Fish material

The feeding trial was carried out at Nofima's Research Station, Sunndalsøra. The feeding trial was performed for 112 days. Atlantic cod (*Gadus morhua*) with an average weight of 479g (max 580g, min 380g), were acclimatized to experimental conditions before the trial started. The fish were distributed randomly to 12 tanks (2-m^2 fibreglass tanks, water depth of 60 cm). 35 fish were put into each tank. The tanks were covered with lids (30 x30cm), where the feeding automat was mounted.

The tanks were supplied with saltwater (33g l^{-1} salinity).Water temperature were kept at 9.9 °C in average (min 7.8°C, max 14.3 °C) during the experiment. The oxygen level in the outlet water was checked once a week. The mean values were in the range of 85-90 % saturation during the first 8 weeks of the experiment. Mean oxygen saturation during the last 9 weeks were in the range of 81-85%, with the lowest single observation of 59 % saturation.

Initial samples of fish (3 x 10 fish) for analyses of whole body chemical composition were taken from the population of fish used in the experiment. The fish were individually weighed at the start and after 112 days, when the experiment was terminated. The fish were anaesthetized using MS-222 (60 mg l^{-1}). Fifteen fish per tank were sampled for different analyses (liver weight was recorded in all these fish). Five additional fish per tank were sampled for whole body proximate chemical analyses, as well as FA composition.

Prior to the experiment, the fish were fed a commercial cod diet (produced by Skretting AS). The fish were offered feed from automatic feeders (disc-feeders). Feeding was done every day with 90 min feeding period. Uneaten feed was collected and separated from feces.

3.2 Experimental diets

In the experiment, four diets were used (extruded pellets), differing in oil contents. The feed was produced by EWOS, Dirdal, Norway. The feed contained yttrium oxide (Y_2O_3) as an inert marker for digestibility determinations. Formulation and proximate composition of the experimental diets

is given in table 1. FAs composition is shown in table 2. Four different types of oils were introduced in the diets: standard fish oil (FO), fish oil and rapeseed oil (FO-RO), a salmon oil of low quality (SO-L) and a salmon oil of high quality (SO-H). The quality of the oils was graded after measuring their TBARS levels (table 3).

The diets were homogenized and analyzed for chemical composition: dry matter (oven drying at 105°C, 16-18 h, to constant weight), total lipid (HCL hydrolysis and petroleum ether extraction in a Soxtec HT-6 apparatus), nitrogen (Kjeltech Auto Analyser, Tecator, Sweden), ash (flame combustion followed by 3-4 h at 550°C until constant weight), and energy (bomb calorimetry; Parr 1271, Parr, Moline, IL, USA). Diets were in addition analyzed for starch (enzymatic method, Megazyme total starch AA/AMG method), and yttrium (ICP spectrometry, as described by Refstie et al., 1997).

Table 1 - Composition of the experimental diets

Diets	**FO-RO**	**SO-L**	**SO-H**	**FO**
Ingredients (g/kg)				
Fish meal	631	631	631	631
Wheat	125	125	125	125
Soy protein concentrate	60	60	60	60
Wheat gluten	60	60	60	60
Fish oil, standard quality	55			110
Rapeseed oil	55			
Salmon oil, high quality			110	
Salmon oil, low quality		110		
Vit /min premix	3.5	3.5	3.5	3.5
Inorg. P	10	10	10	10
Yttrium	0.1	0.1	0.1	0.1
Chemical composition				
Dry matter (dm), %	93.8	93.3	93.4	93.4
Crude protein, % of dm	53.2	53.3	54.1	52.1
Fat, % dm	19,1	17,8	17,2	20,2
Starch, % of dm	8.1	8.5	8.7	8.2
Ash, % of dm	9.2	9.2	9.1	9.1
Energy, MJ/kg	22	21.6	21.5	22.1

All feed ingredients were chosen according to commercial quality standards. Micro ingredients include vitamin and mineral premixes according to NRC standards (NRC, 1993).

Table 2 - Fatty acids composition of diets

Diets	**FO-RO**	**SO-L**	**SO-H**	**FO**
Fatty acids (% of total)				
C 14:0	3.2	3.9	4.3	5.2
C 16:0	12.8	12.3	13.0	17.2
C 18:0	2.1	2.3	2.4	2.4
Σ Saturated [a]	**19.3**	**19.5**	**20.8**	**26.2**
C 16:1 n-7	3.6	4.4	5.1	5.7
C 18:1 n-7	3.3	3.2	3.2	2.7
C 18:1 n-9	29.5	20.8	17.7	17.2
C 20:1 n-9	3.5	6.1	5.7	3.6
C 20:1 n-11	1.3	1.3	1.4	2.1
C 22:1 n-7	0.4	1.1	1.1	0.7
C 22:1 n-9	0.7	1.0	1.0	0.6
C 22:1 n-11	3.5	6.3	6.1	4.3
C 24:1 n-9	0.7	0.6	0.7	1.0
Σ Monounsaturated [b]	**47.9**	**47**	**44.4**	**39.9**
C 18:2 n-6	10.2	7.6	6.8	4.3
C 18:3 n-3	4.5	2.3	1.8	1.9
C 20:4 n-3	0.5	1.1	1.2	0.7
C 20:4 n-6	0.5	0.5	0.6	0.7
C 20:5 n-3	5.8	6.6	7.6	9
C 22:4 n-6	0.2	0.4	0.4	0.4
C 22:5 n-3	0.7	2.3	2.6	1.3
C 22:6 n-3	7.6	8.8	9.8	11.1
Σ Polyunsaturated [c]	**31**	**31.4**	**32.6**	**30.8**
Σ n-3	18.9	20.3	22.1	23.5
Σ n-6	11.2	9.3	8.6	6.0
Σ EPA+DHA	13.4	15.4	17.4	20.1
Σ Detected	98.2	97.8	97.7	97
Σ Identified	99.3	99.2	99.2	98.7

[a] *includes: C 15:0, C 17:0, C 20:0*

[b] *includes: C 15:1, C 14:1 n-5, C 16:1 n-9, C 17:1 n-7, C 16:1 n-5, C 18:1 n-11, C 20:1 n-7*

[C] *includes: C 16:3 n-4, C 18:3 n-4, C 18:3 n-6, C 20:2 n-6, C 20:3 n-3, C 20:3 n-6*

FAs (a, b, c) were not included in the table because the percentages of these FAs were less than 0.5 %.

Table 3 - TBARS level in the feeds oil

Diets	**FO-RO**	**SO-L**	**SO-H**	**FO**
TBARS (µM/g)	4.3	67.2	42.1	8.6

3.3 Growth performance calculation

To evaluate growth performance of experimental fish and the quality of the experimental diet, we used the following formulas:

Specific Growth Rate (SGR):

$$SGR = (e^{((\ln W1 - \ln W0)/d)} - 1) * 100$$

Where w_0 and w_1 are start and final fish weights, respectively and d is feeding days

Thermal growth coefficient (TGC) or growth factor (GF) (Described by Cho., 1992):

$$TGC = 1000^* (W1\ (1/3) - W0\ (1/3)) / DGR$$

Where w_0 and w_1 are start and final fish weights respectively, t is temperature °C and $DGR = \Sigma$ (t*feeding days).

Hepatosomatic index (HSI):

$$HSI = (\text{liver weight/total weight}) \times 100.$$

3.4 Liver homogenization

Homogenization is a process that involves breaking apart calls; releasing organelles and cytoplasm and making the animal tissue uniform.

For tissue homogenization, five fish per tank were used (three tanks per diet). We pooled the fish form each tank, and got three pooled samples per dietary group.

We used three different types of homogenates in the analysis: RIPA buffer homogenate, sucrose buffer homogenate and homogenate with dry ice.

RIPA buffer and sucrose buffer:

- RIPA buffer (R 0278, Cayman chemicals) includes 50 mM Tris-HCl, pH 8.0, with 150 mM sodium chloride, 1.0% Igepal CA-630 (NP-40), 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate. Further the buffer was added a protease inhibitor cocktail (P8340, Sigma). The buffer was diluted 5x (1:4) with water, and added 5 µl of protease inhibitor per 1 ml of buffer.
- Sucrose buffer, includes 0.25 mM sucrose, 15 mM HEPES, 1mM EDTA and 1 mM EGTA.

Performing the homogenization (RIPA and sucrose homogenate)

- ✓ 100 mg of tissue was weighed into a tube. (Precellys plastic tubes with 3 ceramic beads).
- ✓ We added 1 ml of buffer (RIPA or sucrose).
- ✓ The mixture was kept on ice all the time!
- ✓ Homogenization was done with a "tissue homogenizer machine (Precellys), Bertin technologies, France)". A pre-set programme was used (5000 rpm; 2 X 20sec with a break of 15 sec between each round).
- ✓ After homogenization, we centrifuged the samples 10 min, at 4°C, 1600 x g (rcf).
- ✓ The supernatant was transferred to a new tube and frozen down on -80°C until analysis.

RIPA buffer homogenate was used for Thiobarbituric Acid Reactive Substances (TBARS) analysis and sucrose homogenate was used for superoxid dismutases (SOD) analysis.

The tissue homogenate for lipid analysis was done by grinding the tissue with dry ice.

The sample homogenisation with dry ice was done by a blender (nr. 7009G/7009L, Warning Commercial, CT, USA) with a stainless steel mini container.

- ✓ First, liver samples (whole liver) were added to the homogenising container together with dry ice.
- ✓ Samples were grained very well to form homogeneous mass. Note: To get a good fraction division it is necessary to have enough of dry ice.
- ✓ Then, liver homogenates were transferred to plastic bags. It is important to keep bags open for 1-2 days until dry ice evaporates.
- ✓ The samples were stored at -80 °C until analysis.

3.5 Lipid analysis

Liver homogenate, made by grinding liver tissue with dry ice as described above, was used for lipid analysis.

a. Determination of total fat (Folch method) (Folch et al., 1957).

Performing the Folch method

- ✓ Tissue samples were first weighted in duplicate (1.5 g ±10 %) and placed in glass vials.
- ✓ 50 ml of chloroform: methanol solution (2:1) and 12 ml of 0.9% saltwater (NaCl) solution was added to the samples. Note: mixed very well.
- ✓ The mixture was homogenised for 60 seconds with a knife homogenizer and again added saltwater solution (13 ml).
- ✓ Then, the mixture was further homogenised with the knife homogenizer for 30 seconds. The solution starts separating (top and bottom layers). Note: The top layer is the water soluble phase which mainly contains water and methanol, and was not used for analysis.

The bottom layer is the most important part for the further analysis containing the lipids and chloroform.

- ✓ Further, the homogenate was filtered and transferred to glass vials.
- ✓ Vials were capped and placed to a fridge to the next day.
- ✓ The water/methanol part was pipette off and 20 ml of each sample solution (chloroform part) was transferred to a clean glass vials (with a known weight) and evaporated in the air.
- ✓ When chloroform was evaporated off, the vials were placed on an oven with 105°C for 20 min to evaporate possible rest of water in the samples.
- ✓ Then vials was placed to the desiccators and cooled.
- ✓ Vials were weighted and the weight of the total lipid was registered.
- ✓ The residual lipid extract was dissolved in benzene (2ml)

This total lipid fraction of the Folch extract was further used for analysis of total FA composition analysis, total lipid classes and for phospholipid classes.

b. Fatty acid composition analysis

The lipid extract (from above) was further transmethylated over night with 2, 2-dimethoxypropane (0.2ml) and methanolic HCl (2ml) as described by (Mason, & Waller, 1964) and by (Holshi et al, 1973) in order to produce methylesters of the FAs. The next day, 2 ml of hexan and 4 ml of 0.6 % $NaHCO_3$ were added to the samples. Further, the benzene / hexane phase containing the FAs, was pipetted off and evaporated under N_2-gas.The dried fraction of FA methylesters was redissolved in 1.5 ml of hexane prior to gas chromatography (GC) analysis. The methyl esters of the FAs were separated in a GC (Hewlett Packard 6890) with split injection (50:1), SGE BPX 70 capillary column (length 60m, internal diameter 0.25mm and the thickness of the film 0.25µm) and flame ionization detector, and the results were analyzed using HP Chem

Station software. The carrier gas was helium. The injector and detector temperature were 300°C. The oven temperature was raised from 50°C to 170°C at the rate of 4°C/min and then raised to 200°C at the rate of 0.5°/min, and finally raised to 300°C at rate of 10°/min. The relative quantity of each FA present was determined by measuring the area under the peak. The identity of each FA was determined according to standards.

c. Lipid class analysis

Thin layer chromatography (TLC) analysis of lipid classes

The total lipids were analyzed with TLC to investigate composition of the different lipid classes. Glass, high pressure (HP) TLC plates (20x10 cm; silica gel 60; 0.20mm layer, (Merck, Darmstandt, Germany)) pre-coated with silica gel were used. The analysis was performed according to (Olsen & Henderson, 1989) with slight changes. Prior to use, the plates were pre-developed to full length with hexane: diethyl ether: acetic acid (85:15:1) as mobile phase and dried in the air. Then the plates were activated for 1 h at 110°C and stored in a vacuum desiccator until further use. The samples were taken directly from the Folch extract and an amount of 5 µl per sample was applied on the HPTLC plates with a CAMAG TLC sampler 4 (Camag Linomat 5; Switzerland) with an application speed of 250 nl /sec. Nitrogen was used as working gas. All samples were applied in duplicate, and the distance between tracks was 10 mm. The lipids were then separated in a Twin through Chamber 20x20 (Camag Switzerland) using 25ml hexane: diethyl ether, acetic acid (85:15:1) as mobile phase. The chamber saturation was increased by placing a piece of filter paper in the chamber. Plates were removed from the chambers when they were developed for 7 cm from the origin point, and air dried at room temperature. The different lipid classes were visualized by dipping the plate into a glass tank with 3 % copper acetate and 8 % phosphoric acid, followed by charring the plate at 130° C for 4 min. The lipid classes were identified by comparison with commercially available standards, and quantified by scanning densitometry using a CAMAG TLC Scanner 3 (Camag, Switzerland). Scan lines were analyzed using an integrator (WinCats-planar Chromotography, version 1.3.3).

d. Phospholipid class analysis

The Folch extract was also used for PL class analysis. In general, the procedure for PL analysis (separation of PL) was similar to the procedure for lipid class analysis, however the mobile phase was different (mobile phase; chloroform: methanol: water: ammonia (60:34:4:2)). The separation of phospholipids was done in two stages. This was done due to a very high fat level in the liver. The lipid in the liver is mainly unpolar lipids. The unpolar lipids were first removed to give better and easier separation of the PLs.

Stage 1- Removing of the unpolar lipids

The TLC plates (Watmann K6 - Silica Gel 60, 25mm gel thickness, 20x20 cm) were desiccated at 120° C for 20 min before use. Then, the Folch extract solution was evaporated, redissolved in hexan and applied onto TLC plates.

As a mobile phase: chloroform: methanol: water: ammonia (60:34:4:2) was used. During the phase separation, neutral lipids migrate to the top of the TLC plates, while the polar lipids will not migrate, and stay where they were applied. After separation the plates were air dried in the fume hood.

Further, the TLC plates were sprayed with di-chloro-fluorescein solution (2-7-Diklorofluorescin 2 % solution in 95 % ethanol), dried and moved to the dark under ultra-violet light (366 nm). Then, the PLs were carefully scraped from the plates and dissolved in Arvidsons solution (chloroform 500 ml: methanol 390 ml: acetic acid 10 ml: water 100 ml). Finally, the chloroform phase was pipette off and evaporated. The sedimentation phase/pellet was used for the stage 2 separation of PLs.

Stage 2 - Separation of phospholipids

The PL pellet from stage 1 was dissolved in hexane, and applied to TLC plates (20x10cm, silica gel 60, 0.20mm layer, Merch) using a Camag TLC sampler 4(Camag Linomat 5,Switzerland).The different PL classes were then separated in Twin through Chamber 20x20 (Camag, Switzerland) using chloroform: methanol: water: ammonia, 25 % (60:34:4:2) as mobile phase. Finally, plates were dried and put in Cupper (II) sulfate solution and charred at 130 °C for 5 min on a plate

heater. The PL classes were identified by comparison with commercially available standards, and quantified by scanning with DigiStore, Win CATS software.

3.6 TBARS analysis

Thiobarbituric Acid Reactive Substances (TBARS) assay kit (Cayman chemicals, cat. no.10009055) provides a simple, reproducible, and standardized tool for assaying lipid peroxidation in tissue homogenates. The MDA thiobarbituric acid (TBA) adducts formed by the reaction of MDA and TBA under high temperature (90-100 °C) and acidic conditions are measured colorimetrically at 530-540 nm. The measurement of TBARS is a well established method for screening and monitoring secondary peroxidation products of lipid peroxidation.

All reagents were prepared according to manufactured standard.

Colorimetrical Standard Preparation

250 µl of the MDA standard (cat. no.10009202) was diluted with 750 µl of water to obtain a stock solution of 125 µM MDA standard. Then eight clean test tubes were labelled A-H. After that the 125 µM MDA stock solution and water were added to each tube as described in table

Table 4 - Standard preparation

Tube	**MDA (µl)**	**Water (µl)**	**MDA Concentration (µM)**
A	0	1000	0
B	5	995	0.0625
C	10	990	0.125
D	20	980	0.25
E	40	960	0.5
F	80	920	1
G	200	800	2.5
H	400	600	5

Performing the assay

- ✓ First, vial caps were labelled with standard number or sample identification number.
- ✓ 100 µl of sample or standard were added to appropriately labelled 5 ml vials.
- ✓ 100 µl of SDS solution were added to the vials and mixed very well.
- ✓ 4 ml of colour reagent were forcefully added down the side of each vial.
- ✓ Vials were capped and placed in foam holders to keep the tubes upright during boiling.
- ✓ Vials were boiled for one hour. (Vigorously boiling water).
- ✓ After one hour, the vials were immediately removed and placed in ice baht to stop reaction, and incubated on ice for 10 minutes.

- ✓ After 10 minutes, the vials were centrifuged for 10 minutes at 1.600 x g at 4°C. Vials may appear clear or cloudy. Cloudiness will clear upon warming to room temperature. (Vials are stable at room temperature for 30 minutes).
- ✓ We loaded 150 µl in duplicates from each vial to a clear 96-well plate.
- ✓ Absorbance was read at 540 nm in a Titertek Multiskan PLUS MK II plate reader (Labsystems, Helsinki, Finland).

Colorimetric Calculation

- ✓ First we calculated the average absorbance of each standard and sample.
- ✓ Then we subtracted the absorbance value of the standard A (0 µM) from itself and all other values (both standards and samples). This is done to correct for background absorbance ("corrected absorbance value").
- ✓ After that we plotted the "corrected absorbance value" (from the step above) of each standard as a function of MDA concentration and made a standard curve (figure 6).
- ✓ Finally we calculated the value of MDA for each sample from the standard curve.

MDA= ((corrected absorbance)-(y-intercept))/slope

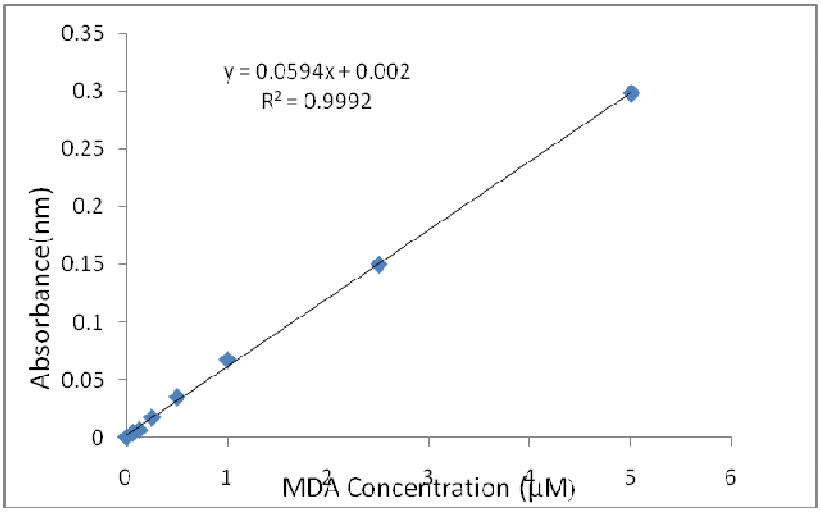

Figure 6 - Representative TBARS standard curve

3.7 SOD analysis

SOD is metalloenzymes that catalyze the dismutation of the superoxide anion to molecular oxygen and hydrogen peroxide and thus form a critical part of cellular antioxidant defense mechanism.

A cell that has a high level of SOD has probably increased production of this enzyme in order to deal with high level of superoxide. To detect the generation of superoxide radicals we used a SOD Assay Kit (Cayman chemicals, cat. no. 706002). Reagents were prepared according to the manufactured standard.

Standard preparation
20 µl of the SOD standard (cat. no.706005) was diluted with 1.98 ml of sample buffer (diluted) to obtain a SOD standard stock solution. Seven tubes were marked from A-G. Then we added the amount of SOD stock solution and sample buffer (diluted) to each tube as described in table 5.

Table 5 - Superoxide dismutase standards

Tube	**SOD stock (µl)**	**Sample buffer (µl)**	**Final SOD activity (U/ml)**
A	0	1,000	0
B	20	980	0.025
C	40	960	0.05
D	80	920	0.1
E	120	880	0.15
F	160	840	0.2
G	200	800	0.25

Performing the assay

- ✓ First, we added 200 µl of diluted radical detector to standard (A-G) and sample wells in a 96- well plate
- ✓ Then we added 10 µl of each standard (A-G) or sample to the plate in duplicate.
- ✓ Reaction was initiated by adding 20 µl of diluted xanthine oxidase to all the well. (Note: adding of xanthine oxidase has to be done as quickly as possible!)

- ✓ The 96-well plate was carefully shaken for a few seconds to mix (covered with the plate cover).
- ✓ The plate was further incubated on a shake for 20 minutes at room temperature.
- ✓ Absorbance was read at 450 nm using a Titertek Multiskan PLUS MK II plate reader (Labsystems, Helsinki, Finland).

Calculations

- ✓ First we calculated the average absorbance of each standard and sample.
- ✓ Then standard A`s absorbance was divided by itself and by all the other standard and sample absorbencies to yield the linearized rate (LR) (LR for std A=Abs std A/abs std A, B= Abs std A/abs std B)
- ✓ Further, we plotted the LR for the SOD standards (from step above) as a function of final SOD activity (U/ml) from table 5. See figure 7 for a typical standard curve.
- ✓ We calculated the SOD activity of the sample using the equation obtained from the linear regression of the standard curve substituting the linearized rate (LR) for each sample. One unit is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical.

SOD (U/ml) = [((sample LR-y intercept)/slope)*0.23/0.01]*sample dilution

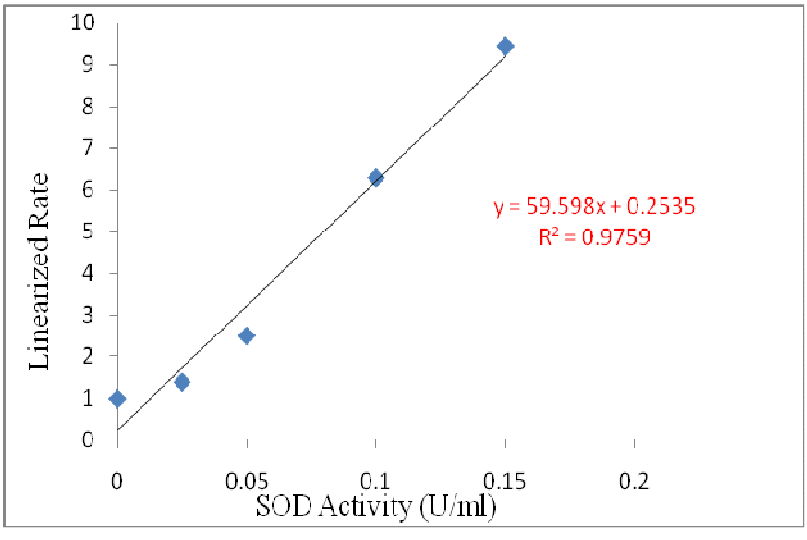

Figure 7 - Representative SOD standard curve.

3.8 Protein analysis

Determination of total protein level was done by a Total Protein Kit (Micro Lowry, Peterson`s modification (Sigma, TP0300)). The procedure is based on two chemical reactions. The first is the biuret reaction, in which the alkaline cupric tartrate reagent complexes with the peptide bonds of the protein. This is followed by the reduction of the Folin and Ciocalteu`s phenol reagent, which yields a purple color. Absorbance of the colored solution is read at a suitable wavelength between 500 nm and 800 nm. The protein concentrations are determined from a calibration curve.

All reagents were prepared according to manufactured standard.

Standards preparation is shown in table 6.

Blank: Lowry + water + phenol reagent

Table 6 - Protein standard preparation

Tube	Protein standard solution (µl)	Water (µl)	Final protein concentration (µg/ml)
1	0	240	0
2	45	195	75
3	75	165	125
4	105	135	175
5	150	90	250
6	210	30	350

Performing the assay

- ✓ First samples were added to test tubes, and diluted with water up to 240 µl (5+235).
- ✓ NaCl was added to give a final concentration of 0.1 M. (24 µl in samples and standards).
- ✓ Then, 24 µl of deoxycholate (DOC) solution was added to each standards and samples (mixed), and left for 10 min in room temperature.

- ✓ After that, 24 µl of trichloroacetic acid (TCA) solution was added to each standard and sample (mixed).
- ✓ A centrifuge step was done at 13000 rpm, 4°C for 10 min to pellet the precipitates.
- ✓ Supernatant was decanted off, and the pellets were dissolved in 240 µl of Lowry reagent solution.
- ✓ The tubes were rinsed with 240 µl of water (mixed), and kept in room temperature for 20 min.
- ✓ Finally, 120 µl of Folin and Ciocalteus Phenol reagent work solution was added to each tube, with rapid and immediate mixing. The mixture was left in room temperature for 20 min for colour developing.
- ✓ We transferred 250 µl in duplicate to wells in a 96- well plate.
- ✓ Absorbance was measured at wavelength 540 nm in a Titertek Multiskan PLUS MK II plate reader (Labsystems, Helsinki, Finland). (Reading must be complete within 30 min).
- ✓ Finally we calculated the protein concentration of each sample from the standard curve.

3.9 Microscopy

Mid section of intestine was used for microscopy. Tissue samples were carefully dissected out from intestine and embedded in tissue-tek / OCT and frozen in liquid nitrogen. The frozen intestinal tissue was preserved at -80°C until analysis.
The frozen samples from the intestine were later processed for histology. Sections were cut about 8-10 µm thick using microtome/cryostat in which the temperature was regulated to -20°C. The sections were mounted on microscope glass and air-dried in room temperature.

a. Proliferating Cell Nuclear Antigen

Intestinal tissue sections were immunolabelled for proliferating cell nuclear antigen (PCNA) (Invitrogen, 93-1143). PCNA expression has a broad correlation with mitotic activity and can be used as a marker for cell proliferation. General process for stem cells proliferation is shown in

figure 8. In general, the stem cell located at the bottom part of the crypt, which is self-regenerating, give rise to new cells. New born cells migrate from the bottom to the top of the microvillus, where they secreted to the lumen.

Cells in mitosis which contain PCNA are stained brown in the nuclei, while non-dividing cells are stained blue.

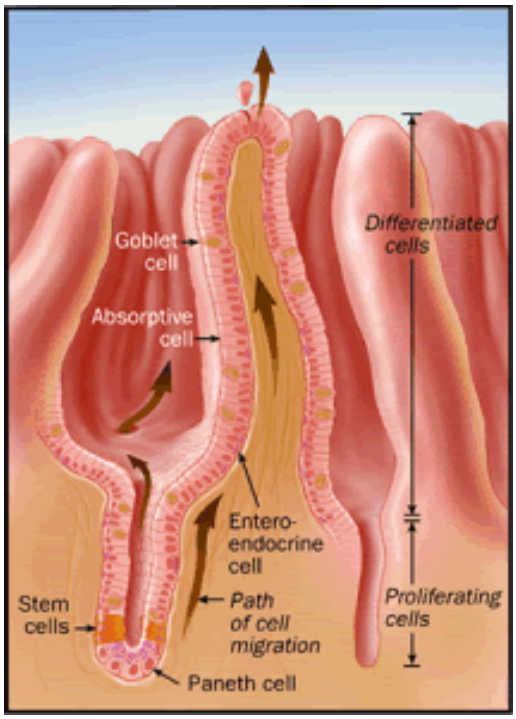

Figure 8 - General process of stem cell proliferation. Picture was adapted from the net (http://medicine.emory.edu/divisions/gi/research/yang_lab.cfm).

The PCNA staining procedure was done according to the manufacturer's protocol.

Performing of PCNA

- ✓ The tissue sample was first blocked with blocking solution and incubated for 10 min at room temperature. (Solution was drained of from the glass).
- ✓ Then, samples were incubated with biotinylated mouse anti-PCNA primary antibody for 60 min and rinsed with PBS solution for 2 min, 3 times.

- ✓ Further streptavidin-peroxidase were applied to the samples and incubated for 10 min and rinsed with PBS solution for 2 min, 3 times. (Tissue was kept in dark).
- ✓ DAB-chromogen was added to the tissue and incubated for 3min. Note: DAB-chormogen was made by adding (1 drop of reagent 4A, 1 drop of reagent 4B and 1 drop of reagent 4C to 1 mL distilled or deionized water).
- ✓ Tissue was incubated in hematoxylin for 2 min, followed by washing in tap water. Then, tissue was kept in PBS solution approximately 30 sec and rinsed with distilled water.
- ✓ Finally, slides were dehydrated in a series of ethanol solution, and cleared with xylene.
- ✓ Samples were mounted with histomount.

The samples were further investigated under different magnifications (10 x and 25 x) with a Zeiss, Axio Observer light microscope.

3.10 Statistical analyses

All the data were subjected to a One-Way analysis of variance (ANOVA) to assess the effect of different dietary oil source. Significant effects were indicated at a 5 % level. The different treatments were ranked using Duncan's Multiple Range Test. Statistical analyses were conducted using the software package SAS System for Windows Release 8.01 (SAS Institute Inc., Cary, NC, USA) or UNISTAT (London, England).

4. Results

4.1 Growth performance

During the experiment we tested four types of feed. The level of protein and starch were the same in all four diets, while fat percentages and the type of oils were different.

Table 7 summarises the growth performance data obtained during the experimental period and HSI obtained at the final sampling. We saw tendency to higher mean final weight of fish fed the SO-H diet (821.2±30.0) than those fed the FORO diet (755.7±6.89), SO-L diet (751.7±27.5) and FO diet (767.8±20.5). However, no statistical differences in growth were observed between the feeding groups. For the other growth parameters (SGR, TGC), there were no differences between the feeding groups either.

The HSI was not affected by lipid composition of the diets. HSI was under 9 in average for all dietary groups.

Table 7 - SGR, TGC and HSI in groups of cod fed different dietary oils.

Diets	**FORO**	**SO-L**	**SO-H**	**FO**	**Anova**
Initial weight	479.5±1.0	478.9±1.30	478.4±0.4	478.7±0.50	0.83
Final weight	755.7±6.80	751.7±27.5	821.2±30.0	767.8±20.5	0.20
SGR	0.4±0.01	0.4±0.03	0.5±0.04	0.4±0.03	0.21
TGC	1.1±0.02	1.1±0.09	1.4±0.11	1.2±0.08	0.19
HSI	8.1±0.60	7.2±0.30	8.9±0.70	8.1±0.40	0.26

Significance level is set at ($p < 0.05$). Data are means ± SEM. SGR=specific growth rate, TGC=thermal growth coefficient, HSI=hepatosomatic index, SEM= mean square error.

4.2 Lipid content, lipid class and phospholipids class composition

The total lipid contents of the livers for all dietary groups are shown in figure 9. There were no significant differences in total lipid content (weight percentage, gram lipid per 100 gram liver) between the dietary groups.

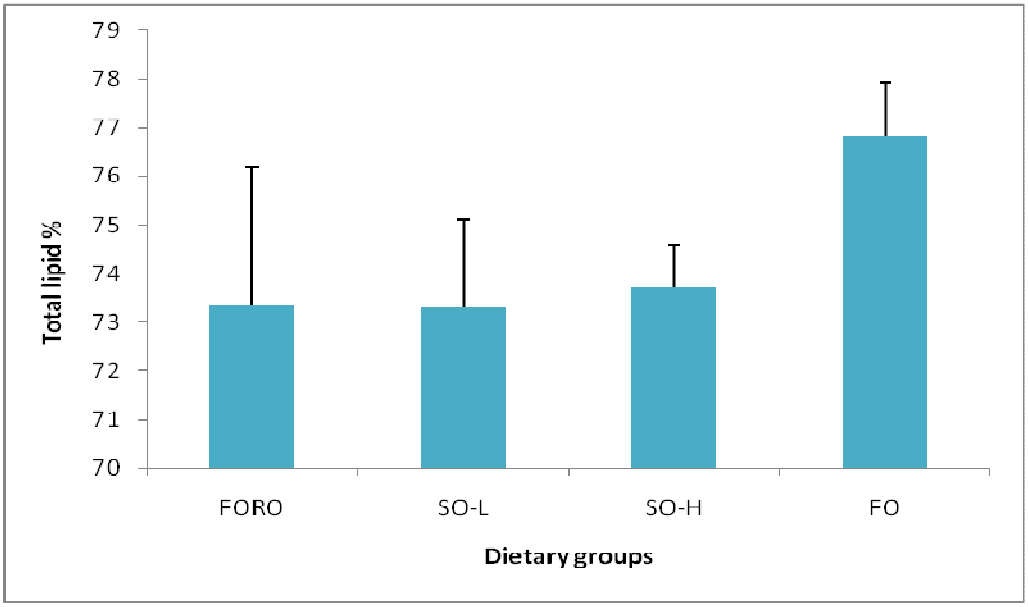

Figure 9: Total lipid content of the liver. Data are means ± SEM, (n=3, each replicate is a pool of five livers). Significance level is set at (p< 0.05). FORO=fish oil and rapseed oil, SO-L=salmon oil low quality, SO-H=salmon oil high quality, FO=fish oil.

The distributions between different lipid classes are shown in table 8. TAG was the dominant lipid class and account for approximately 80 % of total lipids in all dietary groups. Cholesterol ester (CE) constituted around 10 % of total lipids. Cholesterol (CHOL) and PLs were in average 4 % and 3.5 % of total lipids respectively. The diacylglycerol (DAG) constituted around 1.5 % of total lipids. According to the statistical analysis it was no significant differences in lipid class distribution between the dietary groups.

Table 8 - Lipid class composition in cod liver

Diets	**FORO**	**SO-L**	**SO-H**	**FO**
PL	3.9 ± 0.37	3.7 ± 0.11	3.6 ± 0.08	3.4 ± 0.24
DAG	1.4 ± 0.27	2.0 ± 1.36	1.6 ± 065	1.1 ± 0.06
CHOL	4.4 ± 0.60	4.0 ± 0.82	3.4 ± 0.05	4.2 ± 0.36
TAG	80.8 ± 1.20	78.8 ± 1.75	80.6 ±1.81	80.1 ± 1.09
CE	8.6 ± 1.99	11.3 ± 2.31	9.0 ± 1.42	10.3 ± 0.99
FFA	0.8 ± 0.54	0.5 ± 0.33	0.4 ± 0.31	0.6 ± 0.11

Data are shown as percentage of total fat, means ± SEM (n=3, each replicate is a pool of five livers). Significance level is set at (p< 0.05). PL=phospholipids, DAG=diacylglycerol, CHOL=cholesterol, TAG=triacylglycerol, CE=cholesterol ester, FFA=free fatty acids.

The distribution of PLs is shown in table 9. The polar lipids phosphatidylcholine (PC) was the dominating PL class in the liver, and accounted for around 50 % of total PLs, while phosphatidyserine (PS) and phosphatidyletahanolamine (PE) constituted around 20 % of the PLs. The phosphatidic acid (PA) and lysophosphatidylcholine (LPC) constituted around 3.5 % of total PLs. Sphingomyelin (SM) was detected only in the FORO group and was found in low amount. It was not found any significant differences between the dietary groups.

Table 9 - Phospholipid class composition in cod liver

Diets	**FORO**	**SO-L**	**SO-H**	**FO**
LPC	2.7 ± 1.17	3.4 ± 0.0.64	4.5 ± 3.48	2.4 ± 0.38
PA	4.2 ± 1.63	4.5 ± 1.08	4.7 ± 1.63	4.9 ± 0.25
PS	19.0 ± 7.01	19.4 ± 3.16	22.2 ± 2.09	27.2 ± 4.80
PC	55.4 ± 3.97	53.9 ± 1.58	51.4 ± 3.60	49.6 ± 3.02
PE	18.8 ± 5.54	18.7 ± 2.38	17.3 ± 3.68	15.8 ± 1.59
SM	1.3 ± 0.72	Nd	Nd	Nd

Data are shown as percentage of total PLs, means ± SEM (n=3, each replicate is a pool of five livers). Significance level is set at (p< 0.05). LPC= lyso-phosphatidyl-choline, PA=phosphatidic acid, PS=phosphatidyserine, PC=phosphatidylcholine, PE=phosphatidyl-etahanolamine, SM=sphingomyelin.

4.3 Fatty acid composition of the cod liver

The FA composition of the liver (table 10), reflected the FA composition of the diets (table 2). Total percentages of PUFAs were similar for all dietary groups in the tissue. The total amount of n-6 FAs was highest in FORO dietary group. Also SO-L had higher total n-6 FAs compared to FO and SO-L groups, while the total level of n-3 FAs was higher in the FO group than in the FORO, SO-H and SO-L groups. The essential FAs DHA and EPA were highest in the FO dietary group. EPA and DHA FAs were also higher in the SO-H dietary groups compared to the SO-L and FORO dietary groups. Both EPA and DHA were lowest in the FORO dietary group.

The FAs such as 18:1n-9, 18:2 n-6, 18:3 n-3 were highest in the FORO group.

Table 10 - Fatty acid composition of liver

	FORO	**SO-L**	**SO-H**	**FO**
Fatty acid (% of total)				
C 14:0	2.9 ± 0.62[a]	3.1 ± 0.21[a]	3.0 ± 0.38[a]	3.0 ± 0.21[a]
C 16:0	12.0 ± 0.20[a]	12.2 ± 0.25[ab]	12.5 ± 0.24[b]	13.9 ± 0.25[c]
C 18:0	3.4 ± 0.39[a]	3.5 ± 0.28[ab]	4.2 ± 0.38[b]	4.2 ± 0.26[b]
Σ Saturated [A]	19.2 ± 0.40[a]	19.4 ± 0.10[a]	20.5 ± 0.45[b]	22.0 ± 0.36[c]
C 16:1 n-7	4.4 ± 0.34[a]	4.8 ± 0.16[b]	4.9 ± 0.20[b]	5.1 ± 0.17[b]
C 18:1 n-7	4.2 ± 0.14[a]	4.2 ± 0.06[a]	4.4 ± 0.09[b]	4.3 ± 0.01[ab]
C 18:1 n-9	26.1 ± 1.80[b]	21.6 ± 0.67[a]	20.6 ± 0.85[a]	20.5 ± 0.48[a]
C 18:1 n-11	1.1 ± 0.18[a]	1.3 ± 0.25[a]	1.4 ± 0.06[a]	1.1 ± 0.14[a]
C 20:1 n-9	4.9 ± 0.28[a]	6.5 ± 0.08[b]	6.2 ± 0.14[b]	4.9 ± 0.28[a]
C 20:1 n-11	1.3 ± 0.04[a]	1.3 ± 0.03[a]	1.9 ± 0.02[a]	1.7 ± 0.12[b]
C 22:1 n-11	3.4 ± 0.22[a]	4.7 ± 0.15[b]	4.4 ± 0.13[b]	3.5 ± 0.35[a]
Σ Monounsaturated [B]	47.8± 0.92[c]	47.4 ± 0.13[bc]	46.4 ± 0.53[b]	44.0 ± 0.39[a]
C 18:2 n-6	7.9 ± 0.17[d]	6.4 ± 0.06[c]	5.7 ± 0.11[b]	4.5 ± 0.13[a]
C 18:3 n-3	2.8 ± 0.15[c]	1.7 ± 0.05[b]	1.4 ± 0.01[a]	1.5 ± 0.07[a]
C 20:4 n-3	1.1 ± 0.07[a]	1.5 ± 0.05[b]	1.4 ± 0.02[b]	1.0 ± 0.19[a]
C 20:5 n-3	7.3 ± 0.17[a]	7.6 ± 0.36[a]	8.1 ± 0.06[b]	9.5 ± 0.10[c]
C 22:5 n-3	1.2 ± 0.14[a]	2.2 ± 0.13[b]	2.2 ± 0.16[b]	1.4 ± 0.16[a]
C 22:6 n-3	8.8 ± 0.48[a]	9.4 ± 0.36[ab]	9.9 ± 0.46[b]	11.1 ± 0.22[c]
Σ Polyunsaturated [C]	31.3 ± 0.53[c]	31.2 ± 0.28b[c]	31.3 ± 0.80[b]	31.6 ± 0.09[a]
Sum detected	98.3 ± 0.23	98.0 ± 0.25	98.1 ± 0.13	97.5 ± 0.45
Σ n-3	21.9 ± 0.78[a]	22.8 ± 0.36[ab]	23.5 ± 0.68[b]	25.0 ± 0.27[c]
Σ n-6	9.2 ± 0.39[a]	8.1 ± 0.16[b]	7.4 ± 0.15[c]	6.3 ± 0.18[d]
EPA+DHA	16.2 ± 0.50[a]	16.9 ± 0.29[b]	18.0 ± 0.52[c]	20.7 ± 0.12[d]

The percentages of FAs are presented with means ± SEM. Different letters (a, b, c) indicate significant differences ($p<0.05$) between the different dietary treatments.

[A] includes: C 15:0; C 17:0; C 20:0; C 24:0; [B] includes: C15:1; C 14:1n-5; C16:1n-5; C16:1n-9; C17:1n-7; C22:1n-7; C 22:1n-9; C 24:1n-9; [C] includes: C 16:2n-3; C16:2n-6; C16: 3n-4; C18:3 n-4; C18:3n-6; C20:2n-6; C20:3n3; C20: 4n-6; C22:4n-6. Fatty acids (A, B, C) were not included in the table because these percentages were less than 0.5%.

4.4 Oxidative stress markers

a. Thiobarbituric Acid Reactive Substances (TBARS)

The TBARS levels in the livers are shown in figure 10. The TBARS measurement does not show any significant differences between the dietary groups, in despite of tendencies of higher MDA concentration in SO-H and SO-L groups.

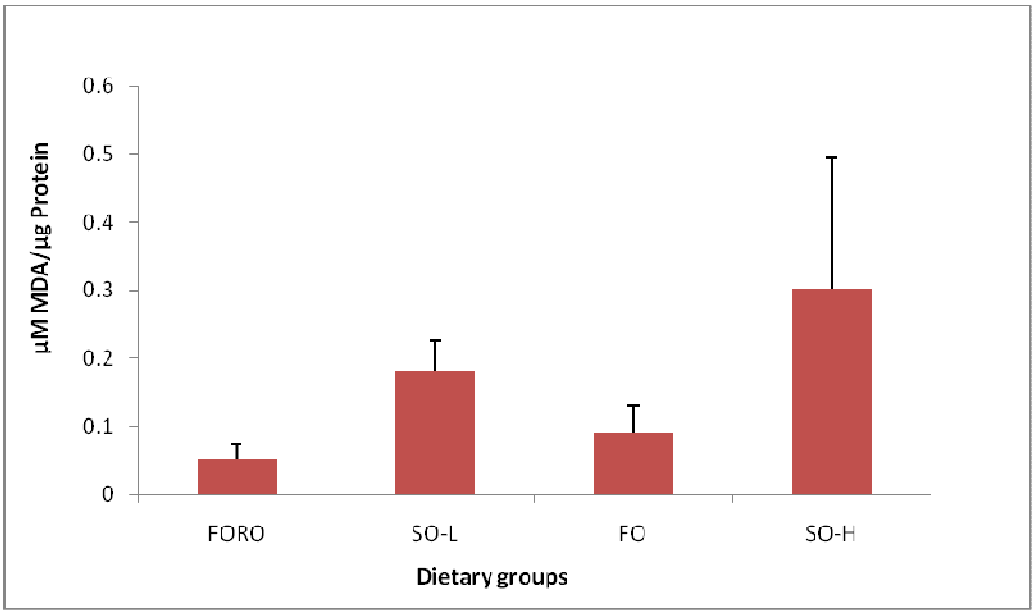

Figure 10 -TBARS activities in cod liver. The results are expresed as µM MDA/µg protein. Data are means ± SEM, n=3, each replicate is a pool of five livers.

b. Superoxide dismutase (SOD)

The result of SOD activity are shown in figure 11. The SOD analysis did not show difference in SOD enzyme activity in the cod livers of any dietary groups, and activities of the enzymes were in acceptable range in all dietary groups. According to the statistical analysis, SOD did not show any significant differences between the dietary groups either.

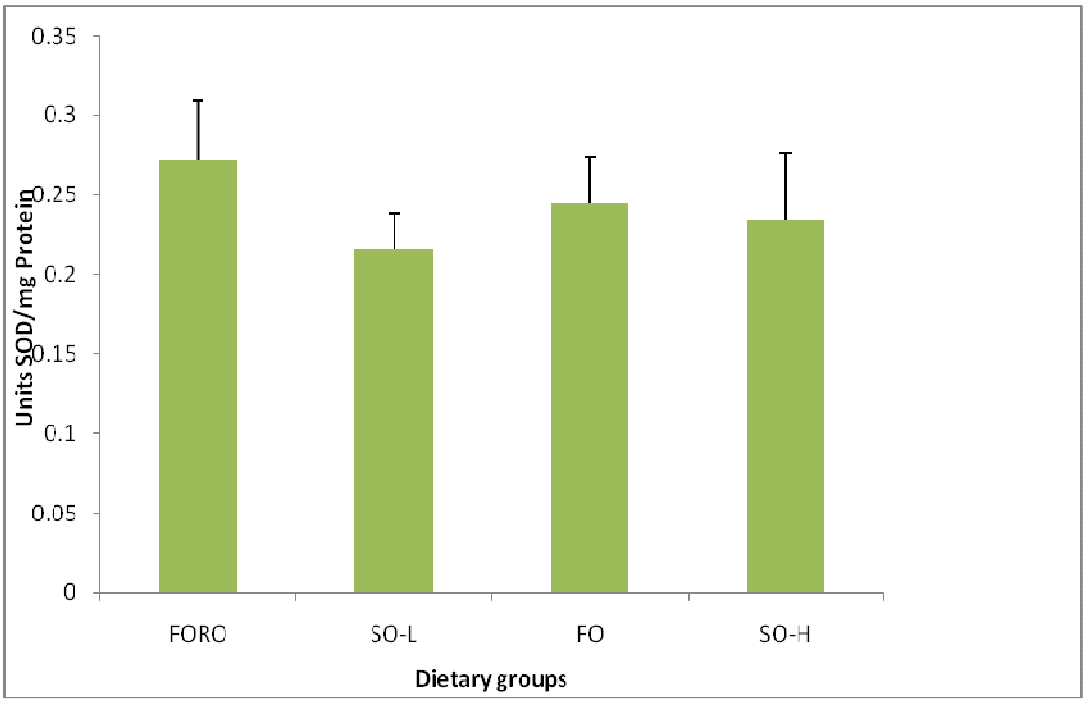

Figure 11 - SOD enzyme activities. SOD was measured in liver homogenates and presented as Units SOD/mg protein. Data are means ± SEM, n=3, each replicate is a pool of five livers.

4.5 Histological examination of cod intestine.

During the light microscopy examination of intestinal sections, no intestinal abnormalities were observed among the dietary groups (figure 12).

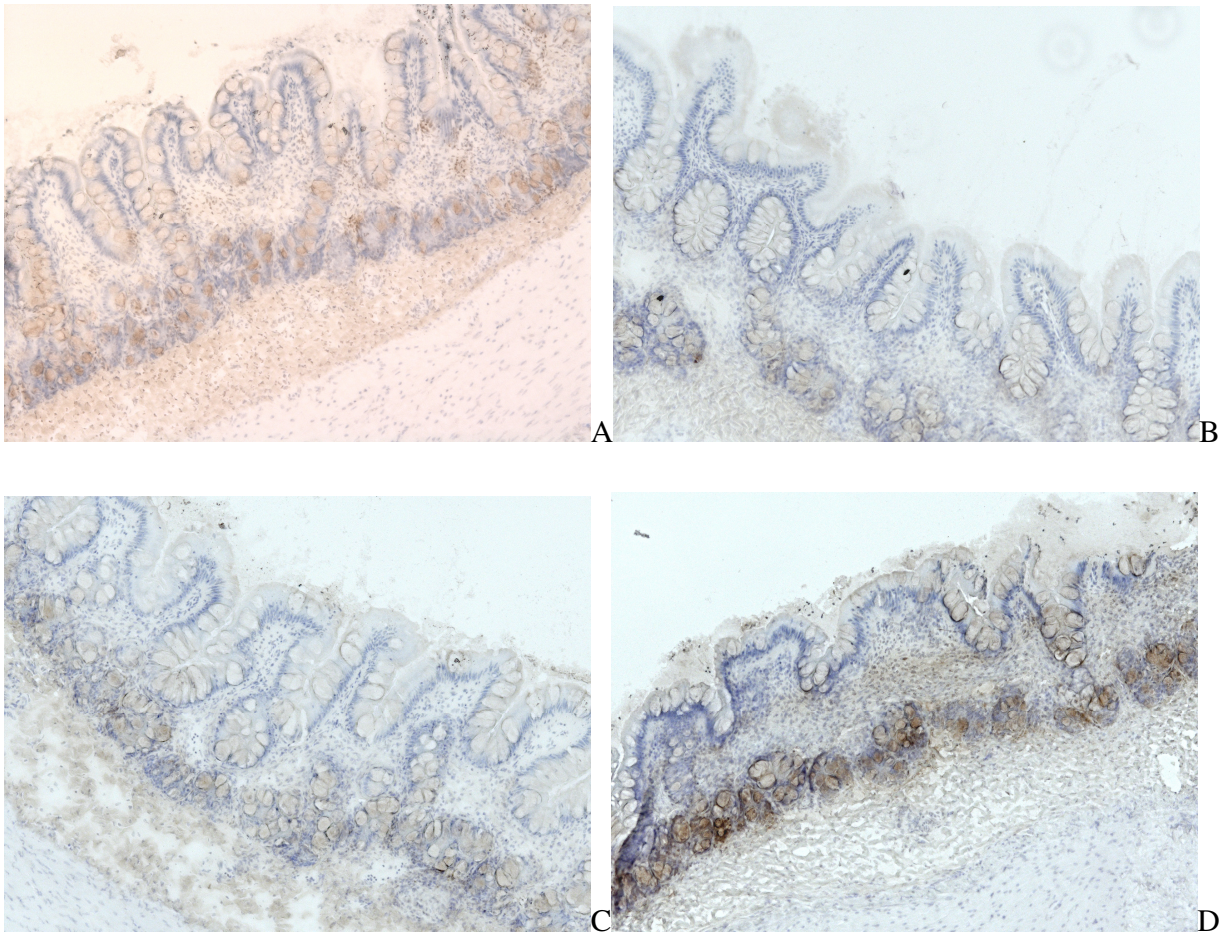

Figure 12 - Light microscopy of cod mid intestine (10 X magnification). The sections were immunostained for PCNA. Cells in mitosis, which contain PCNA, are stained brown in the nuclei, while non-dividing cells are stained blue. The sections were FORO dietary group (A), SO-L dietary group (B), SO-H dietary group (C) and FO dietary group (D). No apparent differences between the dietary groups were seen.

The degree of cell proliferation of the microvillus stem cells was in normal range for all dietary groups in the intestinal sections. Cell proliferation of microvillus stem cells of experimental fish is shown in figure 13.

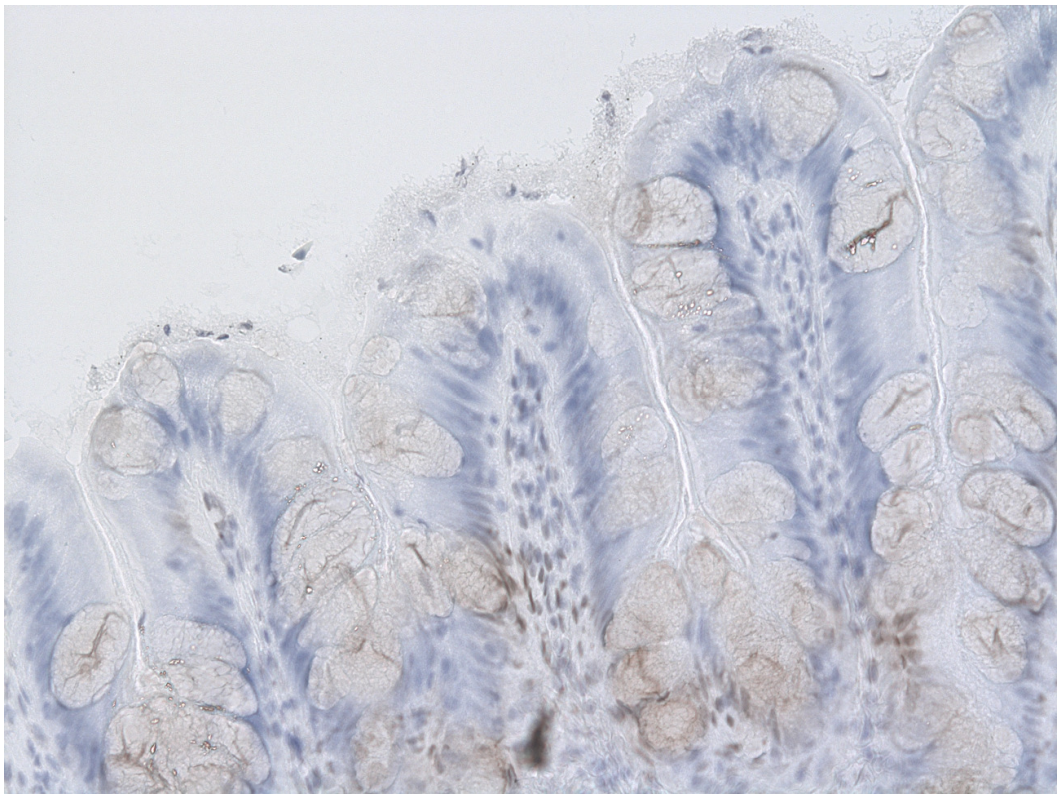

Figure 13 - Light microscopy section of cod mid intestine (25 X magnification). This is a representative picture for all dietary groups. The section was immunostained forPCNA. Cells in mitosis, which contain PCNA, are stained brown in the nuclei, while non-dividing cells are stained blue.

5. Discussion

Feeds are the largest production cost in modern aquaculture. Finding alternatives to the traditional FOs, which are made from small pelagic fish, is a major focus of many researches. Utilization of by-products from the aquaculture industry has received increased attention. Annual discards from the world aquaculture industry is around 20 million tons. This huge amount of discards after fish processing, have potentially high nutritional value if utilized in diets for aquaculture fish. Therefore FO produced from by-products from both fishery and aquaculture may be considered as a good potential future replacement of the traditional FO in diet for cod.

5.1 Growth performance

SO is considered to be a potential important oil source in cod nutrition. In the present work we evaluated SO as an alternative to FO in cod diets. A diet with traditional fish oil was chosen as control. The present experiment showed no differences in growth performance, neither in SGR nor in TGC between the dietary groups. However, we saw relatively large tank variation and individual fish variations in the experiment, which may in part have the result that relatively few statistical differences were found between the dietary groups. One factor which may explain reasons for large individual variation in growth between fish and between the tanks may be that cod aquaculture is still a young industry. The cod has not been through many rounds of genetic selection. The cod used in aquaculture is therefore less domesticated then for instance Atlantic salmon, and thus more vulnerable to stress by environmental factors. The fish in our experiment increased from an initial mean weight of 480 gram to a final weight of 755.7±6.89 for the FORO dietary group, 751.7±27.5 for the SO-L dietary group, 821.2±30.0 for the SO-H dietary group and 767.8±20.5 for the FO dietary group. SGR and TGC were in the range of 0.40-0.48 and 1.38, respectively. Comparison of present results with results from (Refstie et al, 2006) and (Rosenlund et al (2004) for fish with similar parameters showed that the growth performance of fish in the present work was relatively moderate. This may in part due to the lower feed intake found in our study than in the studies, reported by (Hatlen et al, 2007) and (Albreksten et al, 2006).

It has been reported that lipid accumulation in the liver may be directly related to both the dietary level of lipid intake (Lie et al., 1986; Jobling et al., 1991; Kjær et al., 2008a; Hansen et al., 2008) and differences in FA composition of the diet (Ruyter et al., 2006, Kjær et al., 2008c).

The HIS and the lipid content of the liver in the current work were not affected by either the dietary lipid level or the differences in FA composition between the diets. The HSI in average was less than 9 in all dietary groups.

5.2 Lipid class composition

The liver is the major site for lipid storage in cod. The lipid in the liver is mainly unpolar in form of TAG and sometimes accounts for 80 % of total lipids in the body (Griesdale-Hellad et al., 2008; Hansen et al., 2008). In the present study we demonstrated similar results. TAG was the dominant lipid class in the liver and accounted for approximately 80 % of total lipids in all dietary groups, while polar lipids accounted for only 3.8 % of total lipids in average.

Storage lipids in the liver play important roles as a reserve of metabolic energy and they are not always required immediately for physiological function (Sargent et al., 2002).

5.3 Fatty acid composition of the cod liver

The FA compositions of liver lipids were closely influenced by dietary FA input. Similar results have also been reported for other fish species provided with feeds formulated with a variety of lipid sources (Bell et al., 2001, 2003; Mourente & Bell, 2006; Jobling et al 2008). There were clear differences between the FA compositions in the different dietary groups. FAs such 18:1n-9, 18:2n-6 and 18:3n-3 were detected highest in the FORO group. This can be due to high amount of 18 carbon FAs in the plant origin RO. It was demonstrated earlier that vegetable oil may stimulate FAs desaturation and elongation in some fish species (Ng et al., 2007). However, VOs have limitations as the sole lipid sources for cod. Marine fish in general lack the capacity to elongate and desaturete 18:3n-3 to, EPA and DHA, possible due to non functional desaturase and elongase enzymes in the EFA metabolic pathways (Tocher et al., 2003b; Tocher et al., 2006;

Zheng., 2009), which is also the reason why only 50 % inclusion of RO was used in our trial (in order not to create EFA deficiency in the fish). It is also discussed whether feeding diets high in VO might have the potential to reduce human health benefits associating with eating fish, since EPA and DHA are known to have several health beneficiary effects in human (Seierstad et al., 2005; Mourente et al., 2006).

The highest percentage of total n-3 FAs and EPA and DHA was found in the fish given the traditional FO diet, while total n-3, EPA and DHA was lower in the two SO dietary groups. The reason for the lower percentage of n-3 PUFAs in SO, are probably due to the fact that these oils are made from by-products from Atlantic salmon in aquaculture which have been on the diets supplemented with RO. It is quite common today that the lipid fraction of the diet for aquaculture salmon today is based on a mixture of RO and FO. Further, this is reflected in the FA composition of the SO, which are used in the SO-H and SO-L diets and in the FA composition of the experimental fish.

We also saw lower percentages of DHA in the SO-L diet than in the SO-H diet, which may be due to higher degree of lipid peroxidation (higher TBARS value) in the SO-L group. EPA and DHA are the first FAs that are affected by peroxidation, leading to to decreased EPA and DHA percentages. This is probably partly explaining the lower percentage of DHA in the SO-L group compared to the SO-H group.

5.4 Oxidative stress markers

Experimental diets with SO have moderate HUFA level. However, these diets had the highest TBARS level. It was demonstrated earlier that lipids, especially HUFAs, are highly susceptible to oxidative damage (Sargent et al., 2002; Catala, 2008). Some resent studies showed that high levels of n-3 HUFAs in the diets for Atlantic salmon resulted in oxidative stress in the liver (Kjær et al., 2008) and adipose tissue (Todorcevic et al., 2008), and oxidative damage to muscle mitochondria (Østbye et al., 2009).We monitored the oxidative process in the liver of experimental fish through measurements of TBARS and SOD. In our experiment, in despite of

high TBARS in the SO-L and SO-H diets, we did not detect any differences in oxidative process in the liver of the fish in any of the dietary groups.

However, the TBARS results in the liver indicated higher degree of lipid peroxidation in fish given SO. Similar the fish given RO tended to have the lovest TBARS value in the tissue. This can be due to that RO normally contain antioxidants.

5.5 Histological examination of cod intestine

In the current work we had two SO diets; SO-H and SO-L, which had higher degree of lipid peroxidation, than FO and FORO diets. It has been demonstrated that homeostasis and structural features may be disturbed by dietary treatments in the fish intestine (Sanden et al 2005, Olsvik et al 2007, Bakke-McKellep et al 2007). High degree of lipid peroxidation in the dietary groups with SO may contribute to oxidative damage to the cellular mechanism of intestinal tract and stimulate or inhibit cell proliferation. Cell proliferation is often used as an early warning biomarker (Ortego et al., 1995; Sanden et al., 2005).

During light microscopy of intestinal sections, no intestinal abnormalities were observed among the dietary groups. Previous studies have described intestinal cell renewal in teleosts, showing that proliferating cells are found at the base of the intestinal fold (Rombout et al. 1984, Stroband & Debets 1978). We could see some cell proliferation in the basal areas of the intestinal folds; however, this seemed to be the same for all dietary groups. It is however difficult to draw any conclusion concerning dietary affect on cell proliferation in our study, since no statistical analysis was performed due to relatively few samples analyzed in each group. To be able to quantify the degree of proliferation, a higher number of samples need to be analyzed, since there was a big spread between individual fish.

In conclusion, the results of the present thesis suggest that SO can be used as a good replacement for FO in the diets of cod, without negative effect on growth, feed efficiency and health parameters of the fish. It was also demonstrated that the FA composition of the cod may be modified by changing the feed oils.

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