Effects of organic plant oils and role of oxidation on nutrient utilization in juvenile rainbow trout (*Oncorhynchus mykiss*)

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Running head: Organic plant oils in feed for rainbow trout

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Abstract

Producing organic fish diets requires that the use of both fish meal and fish oil is minimized and replaced by sustainable, organic sources. The purpose of the present study was to replace fish oil with organic oils and evaluate the effects on feed intake, feed conversion ratio (FCR), daily specific growth rate (SGR) and nutrient digestibility in diets in which fish meal protein was partly substituted by organic plant protein concentrates. It is prohibited to add anti-oxidants to organic oils, therefore the effects of force-oxidizing the oils (including fish oil) on feed intake and nutrient digestibility was furthermore examined. Four organic oils with either a relatively high - or low content of polyunsaturated fatty acids were considered: Linseed oil; rape seed oil; sunflower oil and grape seed oil. Substituting fish oil with organic oils did not affect feed intake ($P>0.05$), FCR or SGR ($P>0.05$) despite very different dietary FA profiles. All organic plant oils had a positive effect on apparent lipid digestibility compared to the fish oil diet ($P<0.05$), while there were no effects on the apparent digestibility of other macronutrients when compared to the fish oil diet ($P>0.05$). Organic vegetable oils did not undergo auto-oxidation as opposed to the fish oil, and the fish oil diet consequently had a significantly negative effect on the apparent lipid digestibility. Feed intake was not affected by oxidation of any oils. In conclusion, the study demonstrated that it is possible to fully substitute fish oil with plant-based organic oils without negatively affecting nutrient digestibility and growth performance. Furthermore, plant-based organic oils are less likely to oxidize than fish oils, prolonging the shelf life of such organic diets.

Implications

Organic trout farming is a small but growing industry. In near future it is anticipated, that all ingredients in feeds for this segment should be organically approved. Organic plant oils

may be suitable alternatives to fish oil, however, may cause lower utilization and growth due to lack of long chain polyunsaturated fatty acids and may be prone to oxidation as supplementation of antioxidants is not allowed. The tested organic plant oils could all replace fish oil, as they showed a higher digestibility in rainbow trout and were very resistant to oxidation due to presence of high levels of natural antioxidants.

Introduction

Fish meal and fish oil are important constituents of feeds for aquaculture due to the high protein content, optimal amino acid composition, and as supplier of healthy omega-3 long chain polyunsaturated fatty acids (LC PUFAs). Due to global shortage of marine fish meal and fish oil, optimized utilization as well as intensive search for alternative protein and lipid sources in diets for farmed fish is urgently needed. Organic aquaculture is an alternative way of production driven by the growing interest of sustainable utilization of resources (Mente *et al*., 2011). Following the European Union Commission Regulation (EC) No 710/2009 (EU, 2009) for organic aquaculture, detailed rules have been laid down on organic aquaculture production including the compliance with the principles to minimize the use of protein and lipids from unsustainable sources and It is not allowed to add synthetic amino acids to the feed or to use chemically solvent purification methods. Oils from particularly leguminous and oil seed plants are potential sources for replacement of fish oils in aquaculture diets given the steadily increasing production, high availability, relatively adequate nutritional quality and better economic value (Fountoulaki *et al*., 2009). The use of these vegetable oils has gained enormous interest over the past decades. However, most terrestrial vegetable oils possess nutritional drawbacks due to a lack of LC PUFAs. The substitution of fish oil with vegetable oils and the concomitant replacement of fish meal with plant meals may cause essential fatty acid (EFA) deficiency in salmonids.

Furthermore, it may affect lipid deposition, resulting in enlarged livers and an increase in the hepatosomatic index (HSI). A few plants contain considerable proportions of the shorter chain, essential PUFA α-linolenic acid (18:3n-3). Linseed oil in particular but also rapeseed oil are relatively rich in this fatty acid, and both oils are therefore considered applicable alternative candidates to fish oils in feeds for farmed fish.

Oils with a relative high content of PUFAs are more susceptible to oxidation (Koshio *et al*., 1994) than oils characterized by more saturated shorter chain fatty acids, and lipid auto-oxidation may occur during feed processing and storage. The oxidative process is accelerated at higher temperatures, and oxidative changes may cause formation of low-molecular weight carbonyl compounds, which may give rise to unpleasant off flavors known from rancid oils (Kaitaranta, 1992). Dietary supplementation of synthetic antioxidants is not allowed in feed for organic fish (Mente *et al*., 2011), and even though recent studies have investigated the possible use of natural antioxidants more information is needed on the nutritional consequences of auto-oxidation in unprotected organic plant oils. Even moderate lipid auto-oxidation may thus affect the dietary nutritional value as well as nutrient utilization, health and growth of fish (Peng *et al*., 2009). Only few studies have addressed possible additional effects of lipid peroxidation on gustatory palatability and feed intake in fish (Jacobsen *et al*., 1995), despite that the taste of feeds may significantly influence feed consumption (Kasumyan, 1997). In addition to this, the use of different vegetable oils like linseed oil, sunflower oil or rapeseed oil in feed for rainbow trout have caused discrimination between feeds (Geurden *et al*., 2005).

The objectives of this study were therefore twofold: firstly to test the influence of complete substitution of fish oil with either organic rape seed oil, linseed oil, sunflower oil, or grape seed oil on nutrient digestibility and growth evaluated against fish oil as lipid source in

diets in which 47 % fish meal protein was replaced by a matrix of organic plant proteins (Lund *et al*., 2011). Secondly, to investigate the susceptibility of moderate oxidation of these oils, and how this affects the dietary plant oil fatty acid composition, diet palatability (feed intake) as well as lipid digestibility and utilization in juvenile rainbow trout (*Onchorhynchus mykiss*).

Materials and Methods

Diet preparation and force oxidation

Five iso-energetic and iso-nitrogenous diets were formulated by BioMar Ltd, substituting 47% fish meal protein by a fixed matrix of three organic plant protein concentrates as described in Lund et al. (2011) (Table 1). The diets were coated with either commercial fish oil (FO), serving as control diet), or one of the following four cold pressed organic oils: linseed oil (LO), sunflower oil (SO), rape seed oil (RO), and grape seed oil (GO). The diets were prepared by the Danish Technological Institute (Kolding, Denmark) using a twin-screw Werner & Pfleider 37 extruder and fabricated as 3.0 mm pellets. After pelletizing, each batch of pellets was divided into two portions and stored at 2 °C until vacuum coated with oil using a non-industrial coater with variable speed and a maximum of 11.6 psi vacuum. One half of the pellet portion was coated with freshly prepared oils and immediately used in feeding studies. The other half was vacuum coated with force oxidized oils. The latter was obtained by storing the oils in closed buckets for 7 months at room temperature (18-20 ºC). Prior to start of the experiment, the oils were force oxidized by placing them in 1 L glass beakers on an IKA C-MAG magnetic stirrer/hotplate at a light intensity of 3400 lux (OSRAM L Lumilux 36 W/840 warm white) under continuous heating at 48-49 °C. Pure oxygen was continuously added as a slow flow through the oils by glass

pipettes (0.2 L/min for 120 h), slightly modified from a previous description (Koshio et al., 1994).

Experimental design and procedures

Three experimental studies were carried out: 1) A digestibility study to determine the apparent digestibility coefficients (ADCs) of dietary nutrients in the four, non-oxidised organic plant oil based diets and the conventional fish oil control diet; 2) A growth study to evaluate the specific growth rate (SGR) and feed conversion ratio (FCR) of the five diets; and 3) A digestibility study to determine the effects of force-oxidation in nutrient digestibility. The studies were carried out by DTU Aqua (Hirtshals, Denmark) using juvenile organic rainbow trout obtained from Sejbæk Trout Farm (Sjørup, Denmark).

Digestibility and growth study of non-oxidized plant oil diets (experiment 1 and 2)

Experiment 1 lasted 12 feeding days and was designed as a fully random, single factorial experiment with three replicate tanks for each diet (i.e., n=3, 15 tanks in total). Fish with an initial mean weight of 82.2±2.4 g were sorted from a larger batch of fish and randomly distributed among 15, 189 L, cylindrical-conical, flow-through, thermoplastic tanks at a stocking density of 20 fish $tank^{-1}$. The tank setup ensured that all faecal particles were collected in separated sedimentation columns submerged in ice-water as previously described (Dalsgaard and Pedersen, 2011). The tanks were supplied with 10° C tap water at a flow rate of 40 L h^{-1}. A 15 h light: 9 h dark diurnal photoperiod was maintained throughout the trial, and oxygen saturation levels were kept between 70 and 100 % during the experiment. The fish were acclimatized to the experimental conditions and to the diets for 8 days prior to commencement of the experiment. They were individually weighed at the start of the experiment (day 0), and subsequently fed 1.5 % of the estimated biomass d^{-1} (calculated based on an expected FCR) for 9 days. The daily ration was divided into

two equal portions, which were fed at 10:00 and 14:00 h, respectively. Feed waste was registered and counted throughout the trial to derive the exact feed intake. All faeces were collected daily prior to feeding at 10:00 h, and samples from each three consecutive days were pooled (i.e. yielding three faecal sampling periods) and stored at -20 °C until chemical analysis was carried out. Faeces from the second and third sampling periods were analysed for protein, lipid, dry matter (DM) and ash. The fish were individually weighed at the end of the digestibility trial (day 10).

The growth study (experiment 2) was designed as a fully random, single factorial experiment with two replicate tanks for each experimental diet (i.e., n=2, 10 tanks in total). It was carried out in a recirculation freshwater system consisting of 1.18 m x 1.18 m fibre glass tanks with an average water depth of 0.55 m, a mechanical filter (Hydrotech), a submerged biofilter, and a trickling filter (both BioBlok 150-200, EXPO-NET, Hjørring, Denmark). A 14 h light: 10 h dark regime was maintained throughout the experiment. Fish from the same batch as in experiment 1 were randomly distributed among the 10 tanks. The fish were acclimatized to the system and experimental diets for 12 days. The density in each tank was adjusted to 13 kg m^{-3} at the start of the study, and the fish with an initial mean weight of 78.8 ± 12.2 g were fed 1.5 % of the estimated biomass d^{-1} (calculated based on an expected FCR) for 57 days. The feeding period was divided into 3 growth periods of 19 days, each followed by weighing all individuals and adjusting the feed ration. Daily feed waste was collected in swirl unit separators mounted to the tanks and counted to derive the exact feed intake. Dissolved oxygen levels were kept above 70 % saturation at all times during the experiment, and the water temperature was maintained at 15.6 ± 0.5 °C. Ammonium-nitrogen (NH_4-N) was kept below 0.5 mg L^{-1}, nitrite-nitrogen (NO_2-N) below 1 mg L^{-}, nitrate-nitrogen (NO_3-N) between 0-25 mg L^{-1}, and pH ranged between 7.82 and

8.06. At the end of the study the fish were slaughtered for further sensory analyses (Petersen et al., accepted) and dressing percentage (i.e. organs + gastrointestinal content) was measured from 15 fish per tank. Likewise hepasotomatic index (HSI) was calculated from 10 fish per tank (Liver weight / body weight x100).

Digestibility of oxidized plant oil diets (experiment 3)

A digestibility study by use of oxidized oil diets was carried out similarly as described for the non-oxidized diets, but using another batch of rainbow trout. The fish were supplied with 9 º C tap water and a flow rate and oxygen content similar to experiment1. The fish, with an initial mean weight of 103.7±1.5 g, were acclimatized to the experimental conditions for 14 days prior to the study. During the study they were fed 1.3 % of the estimated biomass d^{-1}.

Chemical analysis

Samples of non-oxidized and forced oxidized oils were analysed for fatty acid (FA) composition. Sample preparation trans-esterification and GC-MS fatty acid analyses were carried out as previously described (Lund et al., 2007). Hence, briefly, samples of fish oils and plant oils were collected in pre-weighed glass vials and weighed, followed by trans-esterification by a reagent solution (approximately 1 mL) of acetyl chloride in toluene:methanol (40:50:10, HPLC quality). The fatty acid methyl esters were analysed by gas chromatography-mass spectrometry (GC-MS) on an Agilent 6890 series gas chromatograph equipped with a PTV inlet and an Agilent 5973 mass selective detector. Peaks were quantified by means of the target response factor of the fatty acids relative to a 23:0 internal methyl ester standard from Sigma-Aldrich. Fatty acids are expressed as % of the total fatty acid composition.

Feed samples were homogenized using a Krups Speedy Pro homogenizer and analysed for dry matter and ash (NMKL, 1992), crude protein (ISO, 2005; crude protein = Kjeldahl N x 6.25), and crude lipid (Bligh and Dyer, 1959). Nitrogen-free extract (NFE) was calculated as DM less the sum of crude protein, crude lipid, and ash. All diets were analysed for their oxidation stability and resistance to oxidation by fatty acid analyses: i.e., peroxide value (POV, meq O_2 kg oil^{-1}; (Shantha and Decker, 1994) and anisidine value (p-AnV; AOCS 2009). The peroxide value indicates initial formation of hydroperoxides during early oxidation, the formation accelerates until the decomposition of hydroperoxides outweighs the formation causing POV to decline. The p-AnV method measures spectophotometrically the content of aldehydes during decomposition of hydroperoxides (secondary products of oxidation). The free fatty acid (FFA) content was analysed according to AOAC procedure 940.28 (AOAC, 1995).

Oxidized feed samples were analysed for antioxidants, i.e., α-β-γ-δ tocopherol (E vitamin; AOCS 1990) and ethoxyquin (Ping and Ackman, 2000).

Faecal samples from sampling period 2 and 3 in experiments 1 and 3 were thawed, homogenized using an Ultra Turrax, and analysed for DM, ash, protein and lipid as described for the diets.

Calculations

The apparent digestibility coefficients (ADCs, %) of dietary nutrients and minerals were calculated according to the direct collection method (Jobling, 1994), requiring knowledge of all feed consumed and collection of all faeces produced:

$ADC_i = 100*(C_i-F_i)/C_i$, where *i* corresponds to a dietary macronutrient or mineral (i.e., protein, lipid, NFE or ash), C is the consumed amount of *i* and F is the faecal loss of *i.*

208 The feed conversion ratio (FCR, g g^{-1}) was calculated based on the biomass weight gain
209 and feed intake:

210 FCR = feed intake / weight gain.

211 The specific growth rate (SGR, % d^{-1}) was calculated based on the overall biomass gain in
212 the tanks during the duration of growth:

213 SGR = 100*(ln W_t – ln W_{t0})/Δt, where W_t refers to weight at day t, W_{t0} refers to weight at
214 day t_0, and Δt is the number of days.

215 Hepatosomatic index (HSI) was calculated based on weight of sampled fish and the weight
216 of their liver:HSI = (liver weight / fish body weight) *100.

217 *Statistical analysis*

218 Experimental data were subjected to a single or two factor analysis of variance (ANOVA)
219 or t- test (analytical data) using Sigma Stat 3.5 to detect statistically significant differences
220 between treatment means. In case of two factor ANOVA, dietary lipid origin and oxidation
221 as well as their interactions were used as factors. Levenes test were used to check for
222 homogeneity of variance within the treatment groups, and Holm Sidak all pairwise multiple
223 comparison of means test was applied for testing significance of mean differences
224 between the treatment groups where applicable. Data expressed in percentages were
225 arcsine transformed prior to analysis. The significance level was set at $P < 0.05$.

226 **Results**

227 *Dietary proximate and FA composition*

228 The protein, lipid and NFE content were similar between the five non-oxidized and
229 oxidized diets, respectively (Table 1). However, the protein content was slightly higher in

the non-oxidized diets than in the oxidized diets, while lipid content was highest in the oxidized diets. These minor differences most likely reflected discrepancy in processing techniques during vacuum coating the oils onto the diets.

The total dietary fatty acid content (TFA) was slightly lower in FO than in the plant oil based diets (Table 2). The content of identified fatty acids and fatty acid classes reflected the origin of the oils (Table 2), with fish oil containing more FAs than the plant oils. Linseed oil contained 66% 18:3n-3, sunflower oil and grape seed oil contained 63 % - and 72 % 18:2n-6, respectively, while rape seed oil contained 61 % 18:1n-9. Fish oil was the only oil containing n-6 and n-3 highly unsaturated essential fatty acids (LC PUFAs), including 20:4n-6 (ARA), 20:5n-3 (EPA), and 22:6n-3 (DHA). The fatty acid composition was almost identical between non-oxidized and force oxidized diets, but data revealed slightly lower values of 18:3n-3 in the oxidized LO diet and lower values of 18:2n-6 in the oxidized GO diet. . The main difference observed was a much lower content of DHA in the force-oxidized FO diet compared with the non-oxidized FO diet, which affected the sum of n-3 PUFAs (Table 2).

Feed intake and digestibility in experiment 1 and 2 (non-oxidized diets)

All groups consumed on average >97.8% of the offered feed in both experiment 1 and 2 with no significantly differences (Table 5). The apparent protein digestibility coefficients of both the rape seed oil (RO) and sunflower oil (SO) diets were marginally but significantly higher ($P<0.002$) than that of the linseed oil (LO) diet (Table 3). The lipid digestibility of all plant oil based diets were significantly higher ($P<0.007$) than the lipid digestibility of the fish oil based control diet (FO). In addition, the lipid digestibility of the grape seed oil diet (GO) was higher than that of the sunflower oil diet ($P\leq0.01$).

The digestibility of NFE was low for all diets, presumably due to the high plant concentrate inclusion level (see also Lund *et al*. 2011). It was significantly lower of the LO than of the SO diet ($P<0.005$). Dry matter digestibility, reflecting the sum of protein, lipid and NFE digestibility values, was significantly higher ($P<0.004$) of the SO diet than of the FO diet.

Feed intake and digestibility in experiment 3 (force-oxidized oil diets)

The feed intake varied to some extent between the diets in this experiment (Table 6). Hence, between 17 and 30 % of the feed offered was either not ingested or was rejected, but the differences between dietary treatment groups were not statistically significant ($P=0.154$). The average feed intake was much lower than in experiment 1(i.e. 24%), but a further comparison was not possible due to differences in fish size and feeding level.
The digestibility coefficients of protein were similar between all diets tested ($P>0.05$), while the digestibility of lipid was much lower in the FO dietary treatment group than in four plant oil based diets ($P<0.001$) (Table 4). The apparent NFE digestibility coefficients were similar between the tested oil types ($P>0.05$), and slightly higher than in experiment 1. Dry matter digestibility was lowest in the FO diet ($P<0.001$), consistent with the much lower lipid digestibility for this dietary treatment group. Ash digestibility was lowest for fish on the RO diet as compared with the FO and LO diets ($P<0.01$).

Fish performance (experiment 1-3) and dressing percentage (experiment 2)

Growth rate was higher and FCR was lower in exp. 1 than exp. 3 most likely related to use of different batches of fish and size differences. There were no differences in SGR or FCR between treatments in the digestibility studies (experiment 1 and 3) or in the growth study (experiment 2) ($P\geq0.05$; Table 5 and 6). Dressing percentage (% body weight) was similar

277 in all groups of fish sampled at the end of the growth study (i.e. from 12.9 % ± 1.2 to 14.2
278 % ± 2.1, data not shown). Likewise there were no significant differences (P=0.07) in
279 hepatosomatic index between treatments (Table 5),
280
281 *Antioxidants and autoxidation (experiment 3)*
282 The content of tocopherol (α,β, γ, δ) was highest in the RO diet, followed by the SO, LO,
283 GO, and FO based diets (*i.e*. only oxidised diets analysed) (Table 7). The content of
284 ethoxyquin was relatively higher in the FO diet than in the RO diet and tended to be higher
285 than in the other partly plant based diets consistent with the fact, that ethoxyquin is
286 conventionally added to fish meal and fish oil after processing (Table 7). Main effects of
287 dietary oil source and oxidation could not be properly interpreted due to interactions
288 between the two factors. However, storing the plant oils for 7 months and force-oxidizing
289 them for 5 days seemed to have only minor effects on oil oxidation levels. Hence, each
290 organic plant oil based diet showed no or very minor signs of oxidation as illustrated by
291 almost similar p-AnV and POV values before and after oxidation (Table 7). The fish oil
292 based diet was moderately affected by storage and force oxidation of the fish oil as
293 indicated by higher POV,- p-AnV and FFA values than before storage and oxidation
294 (Table 7).
295
296 **Discussion**
297 *Growth and digestibility*
298 The growth results are in accordance with several previous studies finding no effects on
299 growth or feed utilization of rainbow trout or Atlantic salmon (*Salmo salar*) when partly or
300 completely substituting fish oil with non-organic vegetable oils such as rapeseed oil,

linseed oil, or sunflower oil (Bransden *et al*.., 2003; Bell *et al*., 2004; Petterson *et al*., 2009). To our knowledge, substituting fish oil with grape seed oil in diets for salmonids has not been reported previously.

The improved lipid digestibility of the tested organic vegetable oils as compared to fish oil may have been too marginal (i.e.1.5-2.5 %) to support a measurable effect on growth in the growth study or masked by the formulation of diets optimal in dietary protein content. Dietary methionine level was slightly lower than recommended for rainbow trout (Lund *et al*., 2011), which may also negatively have affected additional protein synthesis and growth in diets with an improved lipid digestibility. Metabolic energy expenditures in fish may increase by elongating and desaturating 18 carbon n-3 and n-6 PUFAs from plant oils to long chain LC PUFAs like EPA and DHA (Geurden *et al*., 2005). However, levels of EPA and DHA in diets fed on plant oils were probably sufficient to sustain physiological requirements in fish as only part of the fish meal (i.e. with a content of 8-10 % fish oil) was substituted by plant protein meals. Long term performance studies have shown that high dietary inclusion levels of vegetable oils may negatively affect growth and feed utilization due to accumulation of lipid droplets in intestinal cells and hepatocytes (Caballero *et al*., 2002). Data on SGR and FCR as well as dressing percentage and HSI were similar between dietary treatment groups in the present growth study, suggesting no such accumulation or differences in lipid deposition and utilization efficiency.

The lipid class and fatty acid composition of the organic plant oils were all within the range previously reported. (The apparent lipid digestibility of all vegetable oils was surprisingly similar taking into account that lipid or FA digestibility may relate to the origin of oils and their chemical and physical properties. The degree of unsaturation, chain length or melting points may thus affect digestive processes (Cabellero *et al*., 2002; Ng., *et al*., 2004), and

different FAs may compete for the same transport mechanism in case of protein-mediated transport (Geurden *et al*., 2009). High levels of saturated FA may negatively affect the formation of micelles in the intestinal lumen and hence reduce the FA uptake by enterocytes (Menoyo *et al*., 2003). As saturated FA in the FO diet comprised approximately 41 % of TFA, far higher than the 7-10% in the vegetable oil based diets, this may potentially explain the relatively lower FO lipid digestibility.

The low NFE digestibility coefficients in exp 1 and 3, reflected the high anti nutritional content in the supplemented plant protein concentrates, which has been previously discussed and reported (Lund *et al*., 2011).

Oxidation of lipids, feed intake and digestibility

Feed intake of the oxidized feed types were lower than for the comparable non oxidized feed types, but direct comparison hampered by use of larger size fish from another batch. The feed intake was not affected by dietary lipid type or by lipid oxidation as the moderately oxidized FO diet was accepted similarly as to the plant oil based diets for which oxidation had limited effect. The effect of oxidization of residual lipids in fish meal on feed intake by rainbow trout has been examined in a previous study, where fresh fish meal was packed in bags with air and stored at 21ºC for 52 weeks prior to incorporating it into the feed (Jacobsen et al. 1995). Feed rancidity caused the fish to reject a significantly higher amount of administered feed (28.6±5.2%) compared to fish fed a diet with non-oxidized fish meal (11.0±3.9%). Peroxide values were 4 times higher in the referred study and the levels of free fatty acids 1.6 times higher than results obtained for the FO based diet in the present study. Differences in methods may explain these results, as FO was stored and oxidized separately from fish meal in the present study. A combination of lower freshness of the fish meal and higher levels of total volatile nitrogen (TVN), combined with

the fact that auto-oxidation and secondary oxidation products may react with amino acids of the fish meal protein causing a reduction in the nutritive quality (Laohabanjong *et al*., 2009) may further have affected palatability negatively in the study by Jacobsen *et al*. (1995).

In the present study auto-oxidation values of the plant oils were only slightly affected by storage for 7 months and subsequent force-oxidization for 5 days, explaining why lipid digestibility seemed not affected. In comparison, lipid digestibility of FO decreased significantly, indicating a potential deterioration of fish oil quality by mild auto-oxidation. The marked decrease in DHA content of the oxidized FO diet may suggest that oxidation caused a transition of n-3 LC PUFAs to other less polyunsaturated derivatives, but changes probably too small to be demonstrated. The decline in the content of DHA in the oxidized fish oil compared to the lack of changes in the content of less unsaturated plant oil FAs are in accordance with previous findings, showing that LC PUFAs are more susceptible to oxidation than less unsaturated FAs due to their chemical structure (Tyl *et al,* 2008; Sun-Waterhouse *et al*., 2011). Thus, oxidized LC PUFAs will result in metabolites with lower unsaturation index and a diminished nutritional value, likely as part of explanation for the negative effect on lipid digestibility.

Previous studies have thus shown that n-3 LC PUFA levels in fish oils decreased following oxidation (Koshio *et al*. 1994; Børsting *et al*., 1994). In the present FO diet DHA was the only LC PUFA to show this decline. Thus, data indicated that measurement of changes in fatty acid composition may be a less sensitive way of assessing oxidative deterioration than for example POV, in accordance with previously suggested (Shahidi and Wanasundara, 2002).

The resistance of organic plant oils to auto-oxidation may be explained by lack of LC PUFAs, as well as the fact that plant oils are known to contain high levels of natural antioxidants such as tocopherol (E vitamin) and phenols protecting the oils. Consistent with this, higher levels of tocopherol were found in the plant based diets (especially RO and SO) comparable to the FO diet. Various antioxidants are used in the food industry as well as supplemented in manufacturing of fish meal and fish oil and may be added to prolong oxidative stability, and are often chemically synthesized (Singh *et al*., 2005; Lutterodt *et al*., 2011). Conventional vegetable oils may be heated, solvent extracted and refined during processing, while in contrast organic vegetable oils are extracted by cold pressing, a method that involves no heat or chemical treatment, and hence may retain a high level of natural antioxidants removing the concern of solvent residues (Lutterodt *et al*., 2011). Consequently, the incidence of feed rancidity may be substantially reduced by the use of organic plant oils. Feeds in which fish meal and fish oils have been replaced by organic plant protein concentrates and plant oils may therefore be stored for longer time and at higher temperatures while maintaining freshness and palatability as compared to fish meal and fish oil based diets. As a further advantage, natural plant phenolic antioxidants possess diverse consumer health promoting properties including antioxidant activity and protection against cardiovascular diseases as opposed to health risks by supplementing synthetic antioxidants (Sun-Waterhouse *et al*., 2011).

In terms of growth performance and utilizationof rainbow trout the present studies suggested that the tested organic plant oils are all candidate alternatives to similar non-organic plant oils or fish oils added synthetic antioxidants. The absence of LC PUFAs in plant seed oils, however, necessitates the use of finisher diets with a certain content of LC PUFAs in order to secure a healthy LC PUFA composition in terms of human dietary

recommendations (Pickova and Mørkøre, 2007), and a more balanced n-3/n-6 ratio due to the high n-6 contents of some of the tested oils (e.g.. SO and GO). Furthermore, data on the slaughtered fish indicated that the vegetable organic oils had significant effects on filet lipid content, fatty acid composition, texture and sensory quality (see Petersen *et al*, accepted).

Conclusion and recommendations

Organic linseed oil, rape seed oil, sunflower oil or grape seed oil may all be potential sustainable alternatives to FO or non-organic plant oils in diets for organic rainbow trout juveniles. The tested plant oils were all very resistant to auto-oxidation, and no deterioration of lipid quality or FA composition was observed, which was probably due to the high content of natural antioxidants. Supplementation of non-organic, synthetic antioxidants may consequently be omitted during diet preparation, which is very promising in terms of use in organic aquaculture. Costs of these organic oils compared to non organic oils are higher, but future scenarios may include production volume and market demands.

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530 Table 1

531 *Diet formulation (% inclusion) and proximate composition (% ww) of the 5 test diets. Values for*

532 *both non oxidized and oxidized diets shown (X/X). Gross energy given as MJ kg^{-} .*

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[1]Diet	FO	LO	SO	RO	GO
Ingredients					
Fish meal[a]	35.2	35.2	35.2	35.2	35.2
Wheat gluten[b]	2.0	2.0	2.0	2.0	2.0
Pea concentrate[c]	16.0	16.0	16.0	16.0	16.0
Horse bean concentrate[d]	15.0	15.0	15.0	15.0	15.0
Rape seed concentrate[e]	10.0	10.0	10.0	10.0	10.0
Fish oil[f]	22.6	0.0	0.0	0.0	0.0
Linseed oil[g]	0.0	22.6	0.0	0.0	0.0
Sunflower oil[h]	0.0	0.0	22.6	0.0	0.0
Rape seed oil[i]	0.0	0.0	0.0	22.6	0.0
Grape seed oil[j]	0.0	0.0	0.0	0.0	22.6
Vitamin and minerals[k]	0.8	0.8	0.8	0.8	0.8
Moisture change	-1.6	-1.6	-1.6	-1.6	-1.6
Proximate composition					
Crude protein	45.6 /43.5	46.0 /42.5	46.0/42.3	45.4/42.1	45.2/42.8
Crude fat	27.9 /28.7	28.9 /30.1	28.7/30.4	28.8/30.0	28.9/29.0
N-free extracts (incl. crude fiber)	13.5 /13.9	13.6 /13.9	13.4/13.6	13.2/13.0	13.4/13.9
Dry matter	95.8 /93.5	97.0 /94.6	96.7/94.3	95.9/93.0	96.1/93.9
Ash	8.7 /8.4	8.6 /8.1	8.6/8.0	8.5/8.0	8.5/8.1
Total P	1.41	1.42	1.41	1.38	1.40
Gross energy	25.2 / 25.7	25.2 / 25.8	25.1/25.9	25.2/25.8	25.3/25.6

[1] Diet abbreviations: FO = fish oil; LO: linseed oil SO: sunflower seed oil; RO: rape seed oil; GO: grape seed oil.

[a f] Sprat (*Sprattus sprattus*), fish meal: low temperature (LT) Supreme, FF, Skagen, Denmark

[b] BioMar, Brande, Denmark

[c] Peas *(Pisum sativum*), Toft Food A/S, Denmark Denmark

[d] Horse beans (*Vicia faba*), DLF-Trifolium A/S, Roskilde, Denmark

[e,i] Rape seeds (*Brassica napus*), Lehnsgaard, Aakirkeby, Denmark

[g] Linseed (*Linum usitatissimum*), Nyborgaard, Vildbjerg, Denmark

[h] Sunflower (*Helianthus annuus*),Urtekram International, Mariager, Denmark

[j] Grape (*Vitis* sp), Earthoil, Bury St. Edmunds, United Kingdom

[k] BioMar, Brande, Denmark, the following was supplied (mg kg^{-1} except as noted): vitamin A 3750 IU; cholocalciferol 750 IU; α-tocopherol, 131.3; thiamine, 7.5; riboflavin, 15; pyridoxine, 7.5; vitamin B12, 0.002; vitamin K3, 7.5; zinc, 75; iodine, 0.9; copper, 3.75; manganese, 22.5; cobalt, 0.75; selenium, 0.19.

553 Table 2.

554 *Total fatty acid (TFA) content (mg g dw^{-1}) and analysed fatty acid (FA) composition (% TFA) of*
555 *non-oxidised and oxidised oils (mean). Fatty acids below 0.1 % are referred to as non-detected*
556 *(n.d.).*

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[1]Diet	FO	FOoxi	LO	LOoxi	SO	SOoxi	RO	ROoxi	GO	GOoxi
TFA	848	803	952	957	911	903	977	985	972	905
FA										
14:0	13.1	13.5	n.d.	n.d.	0.1	0.1	n.d.	n.d.	n.d.	n.d.
16:0	23.5	24.5	4.8	5.3	6.1	5.9	4.5	4.4	6.4	7.1
18:0	2.4	2.6	3.3	3.6	3.4	3.4	1.6	1.5	3.7	4.0
20:0	0.3	0.3	0.1	0.1	0.2	0.2	0.5	0.5	0.1	0.1
22:0	0.2	0.0	0.1	0.1	0.2	0.5	0.3	0.3	n.d.	n.d.
24:0	0.0	0.0	n.d	0.1	0.2	0.2	0.1	0.1	n.d.	n.d.
∑ saturates	41.1	42.4	8.4	9.3	10.3	10.3	7.0	6.9	10.4	11.5
16:1 (n-7)	9.0	8.9	0.1	0.1	0.1	0.1	0.2	0.2	0.1	0.1
18:1 (n-9)	18.1	18.3	12.7	13.6	26.7	26.3	61.3	61.0	16.9	18.1
20:1 (n-9)	17.3	17.5	0.1	0.1	0.1	0.1	1.0	1.0	0.1	0.2
24:1 (n-9)	1.2	1.4	n.d.	n.d.	n.d.	0.0	0.1	0.1	n.d.	n.d.
∑monoenes	45.9	46.3	12.8	13.7	26.9	26.5	62.6	62.3	17.1	18.4
18:2 (n-6)	4.6	4.7	13.1	13.6	62.6	63.0	18.7	19.1	72.1	69.7
18:3 (n-6)	0.2	0.2	n.d.	n.d	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
20:2 (n-6)	0.5	0.4	n.d.	n.d.	n.d.	n.d.	0.1	0.1	0.1	0.1
20:3 (n-6)	0.1	0.2	n.d.	n.d.	n.d.	0.1	n.d.	n.d.	n.d.	n.d.
20:4 (n-6)	0.6	0.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
22:2 (n-6)	0.1	0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
∑(n-6)	6.1	6.1	13.2	13.6	62.7	63.1	18.8	19.2	72.2	69.8
PUFA										
18:3 (n-3)	3.1	2.8	65.6	63.3	n.d.	n.d.	11.6	11.7	0.3	0.3
20:3 (n-3)	0.3	0.3	0.1	0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
20:5 (n-3)	0.3	0.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
22:6 (n-3)	3.3	1.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
∑ (n-3)	7.1	5.2	65.6	63.3	0.0	0.0	11.6	11.7	0.3	0.4
PUFA										
DHA/EPA	9.8	7.5								
ARA/DHA	0.2	0.3								
ARA/EPA	1.8	1.9								
(n-3)/(n-6)	1.2	0.8	5.0	4.7			0.6	0.6	0.0	0.0

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559 [1] Diet abbreviations: FO = fish oil; LO: linseed oil, SO: sunflower seed oil; RO: rape seed oil; GO: grape seed oil. Oxi: oxidised oil equivalent. ARA:
560 arachidonic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid. mean values, n=2

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563 Table 3.

564 *Effects of diets on apparent digestibility coefficients (ADC %) of protein, lipid, NFE and ash in fish*
565 *(experiment 1)*

[1]Diet	FO	LO	SO	RO	GO	Pooled *s.e.*	P value
Crude protein	90.6^{ab}	89.8^{a}	91.0^{b}	91.1^{b}	90.5^{ab}	0.58	*
Crude lipid	94.8^{a}	97.1^{bc}	96.4^{b}	96.3^{b}	97.3^{c}	0.19	***
NFE	10.7^{ab}	6.0^{a}	13.8^{b}	12.3^{ab}	11.2^{ab}	1.50	*
Ash	61.7	62.3	64.4	63.8	63.6	0.61	ns
DM	79.7^{a}	79.9^{ab}	81.3^{b}	81.2^{ab}	81.0^{ab}	0.33	**

566 [1] Diet abbreviations: FO = fish oil; LO: linseed oil, SO: sunflower seed oil; RO: rape seed oil; GO: grape seed oil.

567 † mean values, n=6. ns= non significant (P>0.05) , *, **, *** refer to significance levels P<0.05, P<0.01, P<0.001. Values not sharing a common superscript
568 letter abc in a horizontal row are significantly different (P<0.05)

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584 Table 4.

585 *Effects of diets on apparent digestibility coefficients (ADC %)[†] of protein, lipid, nitrogen free*
586 *extracts (NFE) and ash in fish (experiment 3).*

[1]Diet	FO oxi	LO oxi	SO oxi	RO oxi	GO oxi	Pooled *s.e.*	P value
Crude protein	89.5	90.1	90.3	90.8	90.6	0.59	ns
Crude lipid	86.0^{a}	95.4^{b}	95.7^{b}	96.0^{b}	96.4^{b}	0.21	***
NFE	16.1	19.4	17.4	16.1	21.1	2.34	ns
Ash	70.0^{b}	69.1^{b}	65.9^{ab}	64.6^{a}	68.2^{ab}	0.81	**
DM	78.3^{a}	81.4^{b}	81.1^{b}	81.4^{b}	81.8^{b}	0.38	***

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588 [1] Diet abbreviations: FO = fish oil; LO: linseed oil, SO: sunflower seed oil; RO: rape seed oil; GO: grape seed oil. Oxi: oxidized.

589 [†] mean values, n=6. ns= non significant (P>0.05) , *, **, *** refer to significance levels P<0.05, P<0.01, P<0.001. Values not sharing a common

590 superscript letter abc in a horizontal row are significantly different (P<0.05)

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Table 5.

Effects of diets on feed intake (% of offered feed), feed conversion ratio (FCR) and specific growth rate (SGR, % d^{-1}) in fish shown for digestibility study (exp. 1) and growth study (exp. 2)†. Mean initial and final weight (g $fish^{-1}$), total mortality (% final biomass) and hepatosomatic index (HSI, % body weight) are presented for each diet in growth study.

[1]Diet	FO	LO	SO	RO	GO	Pooled *s.e.*	P values
Digestibility study							
Feed intake	99.7	99.3	95.1	99.2	97.9	1.44	ns
FCR 9 days	0.68	0.69	0.69	0.70	0.71	0.01	ns
SGR 9 days	2.17	2.14	2.09	2.12	2.07	0.03	ns
Growth study							
Feed intake	99.1	98.9	99.2	99.4	99.4	0.68	ns
FCR 57 days	0.92	0.94	0.92	0.91	0.94	0.01	ns
SGR 57 days	1.65	1.63	1.67	1.67	1.59	0.03	ns
Mean weight initial	79.4	79.4	78.4	80.6	78.6	10.8	ns
Mean weight final	204.2	198.9	203.5	199.4	204.1	27.7	ns
Mortality	0.64	0	0.11	0.41	0.63	0.23	ns
HSI	0.89	0.93	0.82	0.94	0.87	0.03	ns

[1] Diet abbreviations: FO = fish oil; LO: linseed oil, SO: sunflower seed oil; RO: rape seed oil; GO: grape seed oil.

† mean values, n=3. ns= non significant (P>0.05).

Table 6.

Effects of diets on feed intake (% of offered feed), feed conversion ratio (FCR) and specific growth rate (SGR) in fish shown for digestibility experiment using oxidized diets (exp 3)[†].

[1]Diet	FO	LO	SO	RO	GO	Pooled *s.e.*	P values
Digestibility study							
Feed intake	69.6	70.1	73.8	82.2	76.1	3.20	ns
FCR 9 days	0.91	0.81	0.82	0.79	0.81	0.03	ns
SGR 9 days	1.22	1.17	1.17	1.49	1.24	0.08	ns

[1] Diet abbreviations: FO = fish oil; LO: linseed oil, SO: sunflower seed oil; RO: rape seed oil; GO: grape seed oil.

[†] mean values, n=3. ns= non significant (P>0.05).

Table 7

Analytical content of (αβγδ) tocopherol (µg/g); ethoxyquin (ppm); peroxide value (POV,meq O_2/ kg oil), anisidine (P-AnV, absorbance units/g of oil) and free fatty acids (FFA, %) in non oxidized and oxidized diets. †

	Diets non oxidized					Diets oxidized						P- values for effect		
[1]Diet	FO	LO	SO	RO	GO	FO oxi	LO oxi	SO oxi	RO oxi	GO oxi	Pooled s.e.	Dietary oil source	Treatment (oxidation)	Oil source vs. treatment
[2]Tocopherol						18.0[a]	36.4[b]	51.5[c]	71.7[d]	25.5[a]	1.09	***		
[3]Ethoxyquin						5.5[a]	4.8[ab]	3.6[ab]	2.9[b]	4.8[ab]	0.34	*		
POV	14.2	23.5	21.1	14.9	15.5	26.3	21.7	28.9	14.8	15.6	1.21	**	*	*
P-AnV	10.4	11.0	9.1	17.1	20.7	144.4	13.3	24.3	20.7	17.1	3.43	***	***	***
FFA	5.1	3.7	2.8	3.2	2.6	6.0	3.5	3.3	2.6	2.5	0.07	***	ns	**

[1] Diet abbreviations FO: fish oil; LO: linseed oil; SO: sunflower oil; RO: rape seed oil; GO: grape seed oil; oxi: oxidised

[2-3] Analysed for oxidized diets only. † mean values, n=2. ns= non significant (P>0.05).*, **, *** refer to significance levels P<0.05, P<0.01, P<0.001. Mean values not sharing a common superscript letter abc in a horizontal row are significantly different (P<0.05)