Hindawi
Aquaculture Research
Volume 2023, Article ID 4001633, 9 pages
https://doi.org/10.1155/2023/4001633

Research Article

Phosphatidylcholine in Krill Meal and Krill Oil as a Source of Choline for Prevention of Intestinal Steatosis in Atlantic Salmon

Kiranpreet Kaur,[1] **Åshild Krogdahl**,[2] **and Trond M. Kortner**[2]

[1]*Aker BioMarine Antarctic AS, Oslo, Norway*
[2]*Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Ås, Norway*

Correspondence should be addressed to Kiranpreet Kaur; kiranpreet.kaur@akerbiomarine.com

Received 14 September 2022; Revised 27 November 2022; Accepted 30 November 2022; Published 7 February 2023

Academic Editor: Houguo Xu

Intestinal steatosis, called lipid malabsorption syndrome (LMS) in severe cases, is a common condition in farmed Atlantic salmon, associated with choline deficiency causing low lipid transport in enterocytes, excessive lipid accumulation, and increased mucosal weight. A previous dose-response study supplying a plant-based diet with choline chloride indicated that 3.4 g/kg choline prevents LMS in Atlantic salmon. However, no similar documentation exists using phosphatidylcholine (PC) as a choline source. The present study therefore aimed to determine the ability of PC from krill meal (KM) and krill oil (KO) towards reducing steatosis in Atlantic salmon. Two diets with suboptimal PC levels (1.5 and 2.6 g/kg) were tested against two control diets, a choline-deficient diet with no supplementation (0.6 g/kg), and a high choline (4.0 g/kg choline chloride) diet. After 8 weeks of freshwater feeding, growth was significantly higher in KM and KO groups, at both PC levels, in comparison to the choline-deficient group. However, growth was significantly higher only in the KM and KO diets with 2.6 g/kg of PC when compared to the positive control. This indicated that suboptimal levels of PC from KM and KO satisfied choline needs for growth. A clear dose-dependent effect on the decreasing pyloric intestine (PI) somatic index was observed for KM and KO diets, with no significant difference between KM and KO diets (2.6 g/kg choline) and high choline reference diet. Accordingly, PC from both KM and KO significantly reduced lipid accumulation in the PI and liver when added to a choline-deficient diet. However, histological and lipid analyses also indicated that the optimal dietary choline requirement for full elimination of lipid accumulation in PI is higher than 2.6 g/kg with KM and KO as supplementary sources.

1. Introduction

Salmon has become one of the most important aquaculture commodities with an annual global growth rate of 7% and Atlantic salmon being the most widely traded global fish product [1]. This growth was driven by easy culture of salmon and its nutritional benefits. Salmon is a rich source of protein, omega-3 fatty acids, minerals, and vitamins [2]. These nutritional benefits and the general health and robustness of salmon depends a lot on the nutrients supplied to the fish through feeds. In the early days of salmon production, the feeds were based mainly on marine ingredients such as fishmeal (FM) and fish oil (FO). However, the increased usage of these limited resources for direct human consumption, for aquaculture as well as for other livestock, unstable supply, and great fluctuations in price have led to a great inclination towards plant ingredients [3]. However, this change in diet composition may result in a deficient and imbalanced supply of essential nutrients, due to negative effects on palatability, and the presence of antinutritional factors on nutrient availability, which may also have other negative effects on the health of the fish [4, 5]. Choline is one such micronutrient, which is present in low concentration in plant-based ingredients in comparison to marine ingredients [6]. Steatosis, in severe cases called lipid malabsorption syndrome (LMS), is a pathological condition associated with delayed lipid transport in the enterocytes, which results in excessive lipid accumulation in the cells, most marked in the pyloric region

[7, 8]. Recently published studies have demonstrated the importance of choline for the prevention of LMS in farmed Atlantic salmon and that choline levels in commercial salmon feeds, until recently, have often been below requirement levels [9, 10]. These studies indicate that a dietary choline level of about 3.4 g/kg is required to prevent LMS [9]. However, the estimated requirement is based on dose studies with choline chloride salt as a choline source, and no corresponding documentation is available for other choline sources such as phosphatidylcholine (PC). The use of synthetic choline sources such as choline chloride may represent certain challenges or limitations during the production and storage of the feed due to choline's strong hygroscopicity and alkalinity. As an example, choline chloride should not be mixed in vitamin premixes [11].

Krill meal (KM) is a sustainable and rich source of PC (40%). KM is obtained from whole Antarctic krill (*Euphausia superba*), which is one of the most abundant species on Earth, with an estimated biomass of around 500 million tonnes, found in the Southern Ocean [12, 13]. Antarctic krill harvesting is highly regulated by the Commission for the Conservation of Antarctic Marine Resources (CCAMLR) [14]. Krill is exclusively harvested in area 48 of the Antarctic peninsula, with a quota set to 1% of the total estimated biomass in that area. Krill plays a key role in the marine food chain in the Antarctic Ocean, and hence, these strict regulations have led to an increase in its biomass over the years, from 60.3 million tonnes measured in 2000 to 62.6 million in 2018/19 [15]. KM is prepared from an extract of ground whole Antarctic krill that is cooked and dried, resulting in a brownish-orange powder. KM contains a well-balanced amino acid profile similar to FM, high levels of phospholipids, n-3 polyunsaturated fatty acids (n-3 PUFAs) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), astaxanthin, vitamins, minerals, and trimethylamine N-oxide. [16]. These nutritional characteristics have made KM an attractive feed ingredient for the aquaculture industry [17]. However, the availability of the PC in KM as a source of choline has not been demonstrated so far. Hence, the present study was conducted with the aim to determine the ability of PC in KM and krill oil (KO) to prevent intestinal steatosis. The strategy was to add the two choline sources, at two doses, to choline-deficient diets and to observe their ability to reduce symptoms of steatosis in the intestine of Atlantic salmon in an 8-week feeding period.

2. Materials and Methods

2.1. Feed Formulation and Characterization. Six diets were formulated (Table 1) including a reference diet with no choline supplement (Ref 0), two diets with KO, and two with KM included at levels which resulted in diets clearly deficient and moderately deficient in choline. The sixth diet was supplemented with choline chloride at a level which gave a choline level above the estimated requirement (Ref high). The feeds were produced by FôrTek, NMBU's experimental feed production unit. All dry ingredients were ground to 0.6 mm particle size before extrusion in a twin-screw extruder, max. temperature 110°C. The pellet size was 3 mm before drying. Yttrium oxide was added to the formulation as a marker for digestibility assessment. After feed production, total choline levels were analyzed for all feeds by Eurofins. In addition, proximate composition and fatty acid profiles were analyzed for all feeds at LabTek, NMBU. Except for the varying choline levels, all diets were formulated to fulfill the requirement of the fish and contained about 38% protein and 22% lipid and were balanced for other nutrients as mentioned in Table 1. The total fatty acid composition of experimental feeds is provided in the supplementary table (S1).

2.2. Fish and Experimental Conditions. The fish feeding trial was conducted at the Centre for Fish Research at the Norwegian University of Life Sciences. The feeding experiment was conducted in accordance with the regulations given by the Norwegian Food Safety Authorities. Atlantic salmon, average weight of 62 grams, were stocked, 35 fish per tank, in 12 cylindrical 250 L fiberglass tanks. The fish were starved for 48 hours after transfer to the tanks to help adaptation to the tank environment. Each tank was supplied with fresh water, and water temperature averaged 13.8°C during the experiment. Dissolved oxygen and pH were measured daily. The water flow rate was 9–10 L/min. Ammonia nitrogen and nitrite levels in the outlet water of each tank were measured once every two weeks. A 24 h light regime was employed. Duplicate tanks were assigned for each test diet. The fish were fed three meals daily, of 30 min duration, during the daytime using automatic belt feeders. The duration of the feeding trial was 56 days.

2.3. Sampling. At the termination of the experiment, 6 fish were randomly taken from each tank and euthanized with an overdose of MS-222. The weight and fork length of individual fish were recorded. The intact intestinal tract of the fish was removed, cleaned of all visceral fat, and divided into 3 regions: pyloric intestine (PI) with the pyloric caeca, stretching from the distal side of the pyloric sphincter to distal-most caecum), midintestine (MI, from the distal side of the pyloric region to the distal intestine), and distal intestine (DI, from the increase in intestinal diameter and ending with the anus). The intestinal segments were opened longitudinally, and the gut contents were sampled into separate Eppendorf tubes, frozen in liquid nitrogen, and stored at −80°C until analysis. The tissue samples collected for histology were fixed in formalin for 24 hours and then stored in 80% alcohol until further analysis. In addition, tissue samples for fatty acid analysis were collected and frozen in liquid nitrogen, transported on dry ice, and stored at −80°C until analysis. The remaining 29 fish in each tank were batch weighed and fed as earlier for an additional 2 weeks for faeces collection, according to the method of Austreng et al. [18]. The fecal samples were pooled by the tank and stored at −20°C prior to analysis.

2.4. Chemical Analyses of Feed, Faeces, and Tissues. The dry matter of the diet samples was calculated after heating at l05°C for 16–18 h. Total nitrogen, carbon, and sulphur were

Table 1: Formulation and chemical composition of the experimental feeds.

	REF0	KO1	KO2	KM1	KM2	Ref high
Ingredients (%)						
Fish meal LT	10	10	10	10	10	10
Soy protein concentrate	20	20	20	15.5	11.1	19.3
Krill meal	0	0	0	6.2	12.3	0
Krill oil	0	1.7	3.4	0	0	0
Corn gluten meal 65%	10	10	10	10	10	10
Vital wheat gluten	10	10	10	10	10	10
Wheat	20	20	20	20	20	20
Fish oil	18	16.3	14.6	16.3	14.6	18
Rapeseed oil	6.1	6.1	6.1	6.1	6.1	6.1
Choline chloride	0	0	0	0	0	0.7
MCP	1.9	1.9	1.9	1.9	1.9	1.9
Limestone	0.4	0.4	0.4	0.4	0.4	0.4
L lys	1.2	1.2	1.2	1.2	1.2	1.2
L met	1.2	1.2	1.2	1.2	1.2	1.2
L trp	0.06	0.06	0.06	0.06	0.06	0.06
L arg	0.04	0.04	0.04	0.04	0.04	0.04
L thr	0.18	0.18	0.18	0.18	0.18	0.18
L val	0.3	0.3	0.3	0.3	0.3	0.3
Stay C 35%	0.1	0.1	0.1	0.1	0.1	0.1
Y_2O_3	0.01	0.01	0.01	0.01	0.01	0.01
Premix	0.5	0.5	0.5	0.5	0.5	0.5
Analyzed/estimated the composition of the experimental diets (%)						
Dry matter	94.7	90.6	94.2	92.1	95.3	93.7
Crude protein (CP)	39.4	36.6	40.4	38.1	39.0	39.7
Fat (total FAs)	20.3	22.1	22.4	20.4	23.4	21.8
Starch	17.7	16.4	16.2	15.3	17.1	16.7
Ash	4.9	4.6	5.1	5.0	5.7	4.8
Gross energy (MJ/kg)	22.5	22.1	22.9	22.0	23.2	22.7
CP/gross energy (g/MJ)	17.5	16.6	17.6	17.3	16.8	17.5
EPA + DHA	24.4	27.6	28.2	25.3	31.3	26.5
n-3/n-6	1.3	1.3	1.3	1.3	1.4	1.3
Estimated choline in supplements mg/Kg	0	1000	2000	1000	2000	3500
Analyzed total choline (mg/kg)	570	1530	2600	1420	2590	3970

analyzed by the combustion method Dumas (ISO 16634 (2008)) using a Vario El Cube elemental analyzer (Elementar Analysensysteme GmbH, Hanau, Germany), fatty acid composition by FAME analysis in a Trace GC Ultra with the auto injector (Thermo Scientific), and yttrium by inductivity coupled plasma (ICP) mass-spectroscopy as described by Refstie et al. [18]. Total choline levels were analyzed by Eurofins Food & Feed Testing Norway. Pooled fecal samples were lyophilized and homogenized.

2.5. Histological Evaluation of Pyloric Caeca and Liver Tissue. Histological sections were obtained from 6 fish/tank or 12 fish/diet. Histological sections were prepared following standard histological methods with sectioning and staining being conducted at the Veterinary Institute histology laboratoryin Oslo, Norway. Tissue sections of the pyloric intestine/pyloric caeca (PI) were evaluated by light microscopy with a focus on changes in the vacuolization of the intestinal epithelial cells. Additionally, the PI sections were evaluated for the presence of specific pathological changes such as inflammation, degeneration, or hemorrhage. Evaluation of the liver sections focused on changes to hepatocyte morphology and the presence of specific pathological changes such as hepatocyte vacuolization, degeneration, hemorrhage, or inflammation. The degree of change for the different morphological characteristics evaluated for the PI and liver was graded using a scoring system with a scale of 0–4 where 0 represented normal, 1, mild changes, 2, moderate changes, 3, marked changes, and 4, severe changes. The histological evaluation was conducted blind.

2.6. Calculations and Statistical Analyses. The thermal-unit growth coefficient (TGC) was calculated as follows:

$$\mathrm{TGC} = 1000 * \left(\frac{\mathrm{FBW1}}{3} - \frac{\mathrm{IBW1}}{3}\right) \mathrm{x}(\Sigma \mathrm{D}), \quad (1)$$

where IBW and FBW are the initial and final body weights and ΣD° is the thermal sum (feeding days × average temperature in °C).

The specific growth rate (SGR) was calculated using the tank means for initial body weight.

IBW and final body weight (FBW) are given as follows:

$$\mathrm{SGR} = \left[\frac{(\ln \mathrm{FBW} - \ln \mathrm{IBW})}{\text{number of da ys}}\right] \times 100. \quad (2)$$

Organo-somatic indices were calculated as percentages of the weight of the organ in relation to body weight.

Apparent nutrient digestibility (ADC) was calculated as follows:

$$\mathrm{ADC} = 100 * \left(\frac{1}{\mathrm{Md}} * \frac{\mathrm{Mf}}{1} * \mathrm{Nf} * \mathrm{Nd} - 1 \right), \quad (3)$$

where Md and Mf represent the concentration of the inert marker in the diet and faeces and Nd and Nf represent the concentration of individual nutrients or energy in the diets and faeces, respectively.

Differences in histological scores for the various evaluated morphological characteristics were analyzed for statistical significance using ordinal logistic regression run in the R statistical package (version 3.6.2; 2019) within the RStudio interphase (version 1.2.5033; 2019). Differences were examined based on odds ratios of the different feeding groups having different histology scores compared to the reference diet (Ref 0). The other datasets were analyzed for statistical significance using a one-way ANOVA with a mean tank value of 6 individual fish as a statistical unit ($n = 2$). Multiple comparisons between the groups were further analyzed using Fischer's LSD test. The level of significance was set to $p < 0.05$.

3. Results

3.1. Growth Performance. Fish fed the Ref 0 diet showed significantly lower growth rates (SGR and TGC) than fish fed the choline-supplemented diets (Figure 1) among which the Ref high diet showed an intermediate growth rate. The diets with KO and KM gave the highest growth rates.

3.2. Digestibility. Digestibility of total choline, crude protein, individual fatty acids, as well as the sum of fatty acids, were significantly higher in all the choline-supplemented diets in comparison to Ref 0 diet and did not differ significantly between the KO, KM, and the Ref high diets (Table 2).

3.3. Organosomatic Indices. The relative weights (somatic index: SI) of PI, MI, and DI, as well as for the liver are shown in Figure 2. Fish fed the Ref 0 diet showed the highest indices of all the intestinal sections, followed by those fed the diets with a low level of PC from krill (KM1 and KO1), which showed higher indices than those fed the suboptimal level of PC from krill (KM2 and KO2). For both choline sources, the PI indices decreased by 0.7–0.8 units per 1000 mg increase in the choline level from about 500 mg to 2500 mg. Fish fed the Ref high diet, containing about 4000 mg, showed similar indices as the KM2 and KO2 groups. Regarding the hepatosomatic index (HSI), significantly higher values were observed for fish fed the KM2, KO2, and Ref high diets in comparison to KM1, KO1, and Ref 0 groups.

3.4. Histological Evaluation. The histological results for the pyloric caeca tissue are presented in Figures 3 and 4. The highest vacuolation scores, characterized as severe steatosis, were assigned to all fish sampled from the Ref 0, KO1, and KM1 groups. Significantly lower average scores were observed in fish fed KM2 and KO2 diets, whereas fish fed the Ref high diet showed the lowest scores. Regarding symptoms of inflammation in the pyloric caeca, mild to moderate symptoms were observed in at least one fish in all groups except for Ref 0. However, no significant diet effect was revealed. For liver tissue, 78% of all the 96 fish were observed with severe hepatocyte vacuolization. No significant diet effects were revealed (Figure 5).

3.5. Fatty Acid Content in Pyloric Caeca and Liver Tissue. Fish fed the Ref 0 diet showed the highest level of the total as well of individual FA in the PI tissue, those fed the low PC diets (KO1 and KM1) showed intermediate levels, whereas fish fed the KO2, KM2, and the Ref high diets showed the lowest fat content. The fat content in the liver showed a similar trend, with the Ref 0-fed fish showing significantly higher hepatic lipid levels than fish fed the choline-supplemented diets (Figure 6).

4. Discussion

The present study was conducted to determine the ability of PC from two krill products, KM and KO, as a source of choline and for the prevention of intestinal steatosis in Atlantic salmon. Two doses of PC from KM and KO, one low (1.5 g/kg) and one suboptimal (2.6 g/kg), were tested against two control diets, one unsupplemented, choline-deficient (0.6 g/kg choline), and one supplemented with an inorganic choline source (choline chloride) to a level above the suggested optimum requirement, i.e., 4.0 g/kg, in an 8-week freshwater feeding trial. The results showed that the PC from both KM and KO, to a similar degree, reduced symptoms of steatosis: a significant reduction in PISI and reduced fat in PI. In addition, liver fat was significantly decreased in both KM and KO diets when compared to the choline-deficient diet.

The growth of the fish fed with KM and KO diets, at both low (1.5 g/kg and 2.6 g/kg) PC levels, was significantly increased compared to the growth in fish fed the unsupplemented choline-deficient diet. It indicated that suboptimal levels of PC from KM and KO included in the diet, with choline levels of 1.5 g/kg, satisfied the needs for growth. The positive effect on growth with KM and KO was most probably due to the increased lipid and choline digestibility, which has been demonstrated earlier in a study by Krogdahl et al. [10], where PC supplementation significantly increased the lipid digestibility (elevated by 2.4%) when compared to a low fish meal unsupplemented control group [10]. This effect was most likely attributed to the role of PC in emulsification of lipids in the stomach and intestine and its role in micelle formation following hydrolysis [20]. Hence, higher lipid digestibility from PC could have led to significantly higher growth with KM and KO in the present study despite the suboptimal level of PC. However, the reason for lower growth in fish fed the Ref high, when compared to KM and KO diets, cannot be explained in the present study as the lipid digestibility was similar in KM, KO, and Ref high

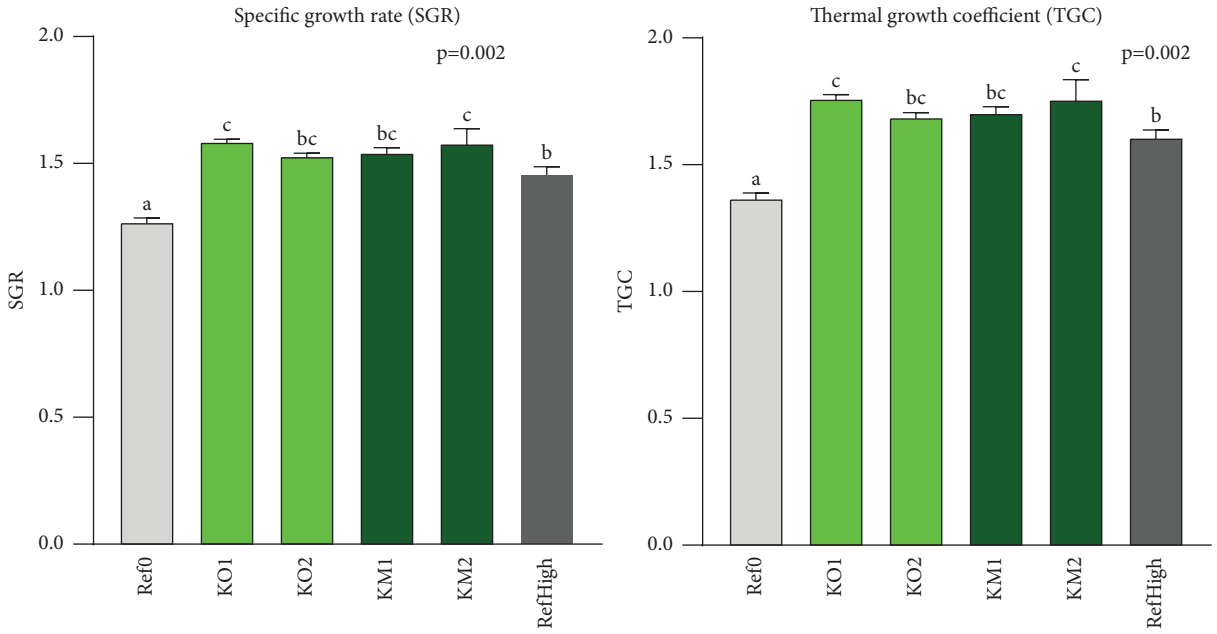

Figure 1: The specific growth rate (SGR) and Thermal growth coefficient (TGC) of fish after 8 weeks of feeding on respective test diets. The SGR and TGC were significantly higher in the choline-supplemented diets in comparison to Ref 0, with the most significant growth observed in KM2 and KO1 groups.

Table 2: Digestibility (%) of choline, crude protein, lipid (sum of fatty acids), and fatty acids*.

	Ref0	KO1	KO2	KM1	KM2	Ref high	p (ANOVA)
Choline	77^{a}	93^{bc}	96^{bc}	92^{b}	95^{bc}	98^{c}	0.0003
Crude protein	93	93	94	95	94	94	0.35
∑ FFA	82^{a}	98^{b}	99^{b}	98^{b}	99^{b}	99^{b}	0.003
C14: 0	77^{a}	97^{b}	99^{b}	97^{b}	99^{b}	99^{b}	0.0006
C16: 0	71^{a}	94^{b}	98^{b}	98^{b}	98^{b}	98^{b}	0.0008
C16: 1n7	88^{a}	99^{b}	100^{b}	100^{b}	100^{b}	100^{b}	0.007
C18: 0	69^{a}	94^{b}	97^{b}	98^{b}	98^{b}	98^{b}	0.0007
18: 1n9c	84^{a}	99^{b}	99^{b}	99^{b}	99^{b}	99^{b}	0.008
C18: 2n6c	89^{a}	98^{b}	98^{b}	98^{b}	99^{b}	99^{b}	0.018
C18: 3n3	90^{a}	99^{b}	100^{b}	100^{b}	100^{b}	100^{b}	0.014
C20: 1	79^{a}	98^{b}	99^{b}	99^{b}	99^{b}	99^{b}	0.0007
C20: 5n3	97	99	99	99	99	100	0.050
C22: 6n3	93	99	99	99	99	99	0.050

*For explanation of diet codes, see Table 1. Different letters denote significant differences among the diet groups.

groups. Interestingly, the growth results from the present study contrasted with the study of Krogdahl et al. [10], where the effects of PC from soy protein concentrate were similar to choline chloride supplementation in Atlantic salmon, investigating similar biomarkers as in the present study. This could have been due to the different sources of PC used in these studies. KM and KO were used as a PC source in the present study as opposed to soy protein concentrate as a PC source in Krogdahl et al. [10]. KO is a richer source of PC (>80% PL) when compared to soy lecithin (62% total PL). Besides, KO has a high proportion of n-3 PUFAs, with an average of 12% EPA and 6% DHA, whereas soy lecithin is rich in n-6 PUFAs linoleic acid (40.2%) and contains oleic acid (10.6%), which is an n-9 monosaturated fatty acid, and n-3 fatty acid linolenic acid (2%) and does not contain EPA and DHA [21]. Higher growth has generally been observed for PL provided by krill over soy lecithin at various PL doses for the first-feeding stage of salmon, where PL from KO was found to be more effective than fluid soy lecithin [22]. In addition, KO was found to be more effective in reducing intestinal steatosis in smaller salmon (2.5 g salmon) and for preventing vertebral deformities [22]. Besides, these effects of better growth and reduced lipid accumulation in the intestine and liver with KM and KO in comparison to soy lecithin have been documented in juvenile stages of other marine fish species such as seabream [23, 24]. The results of the previously mentioned studies suggest that the KO had a better effect in enhancing growth and reducing steatosis when compared to soy lecithin. However, further studies are warranted to determine whether KO effects on growth and lipid accumulation were associated with PL or choline supplementation.

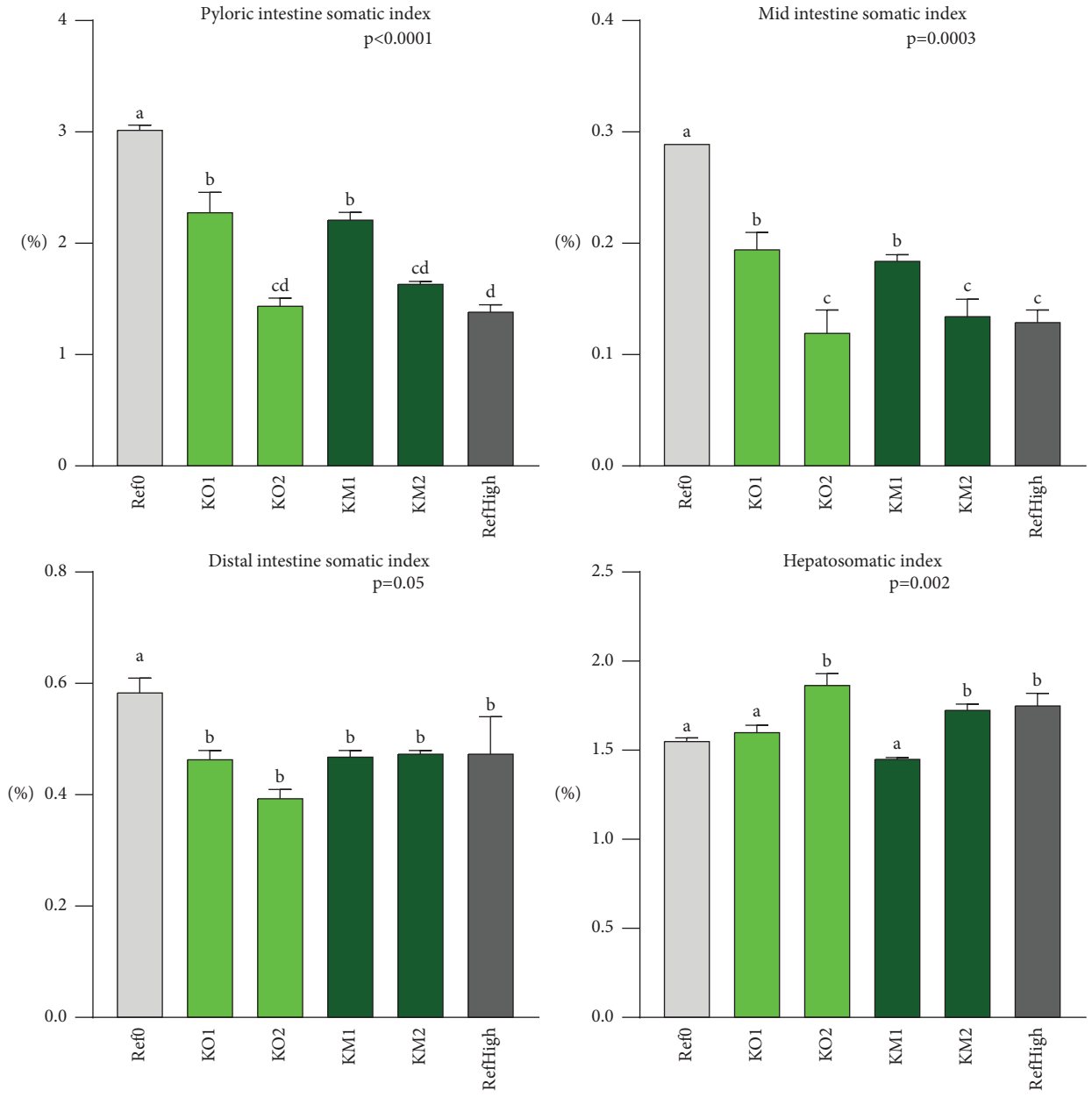

Figure 2: The somatic indices of the intestine and liver of fish after 8 weeks of feeding on their respective test diets. The somatic indices for the intestine reduced significantly with choline supplementation in comparison to Ref 0 group, with the most significant reduction observed in KM2, KO2, and Ref high groups. The hepatosomatic index increased with choline supplementation when compared to Ref 0.

A clear dose-dependent, decreasing effect on the PISI and MISI was observed for both KM and KO diets. At their highest inclusion levels, both krill products (KM2 and KO2) reduced the PISI and MISI to the same level as the high choline reference diet (Figure 2). Similar dose-dependent effects were observed for KM and KO towards significantly reducing the vacuolization (Figure 3) and fat accumulation in PI and liver tissues (Figure 6). The reducing effects of increasing choline supplementation on the PI and MI somatic indices, histological symptoms of steatosis, and lipid accumulation were in line with the results from Hansen et al. [9], where choline chloride was used as a choline source. However, the results of Hansen et al.'s study indicated that a supply of 1000 mg/kg of choline to diets, with similar deficiency as in the present study, would reduce the PI index less than (about 0.4 units) what was observed in the present study. The cause of this difference could be, at least partly, related to differences in feed intake and need for lipid transport, as indicated by the difference in SGR (<1.0 in Hansen et al.'s work, and >1.4 for all supplemented diets in the present study). Hence, the higher growth with KM and KO diets in the present study could have led to different levels of choline needed for the lipid transport when compared to Hansen et al. [9]. However, studies with higher doses of PC from KM and KO are warranted to explore this possibility further.

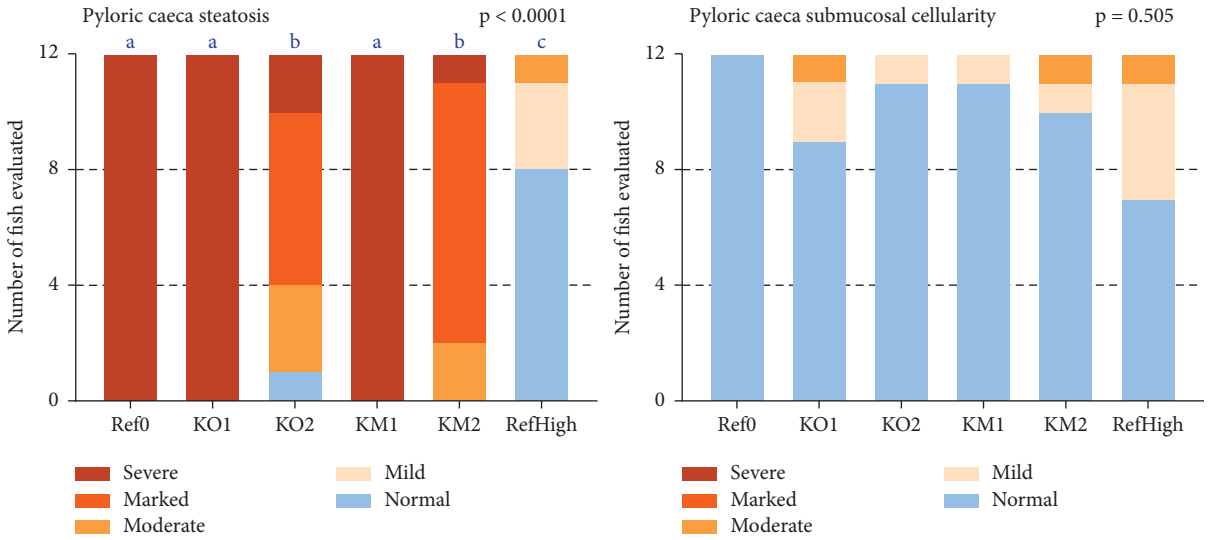

Figure 3: The histological scores for pyloric caeca of fish after 8 weeks of feeding on respective test diets. The scores were significantly lower in the choline-supplemented diets than in Ref 0, with the lowest scores observed in the Ref high group.

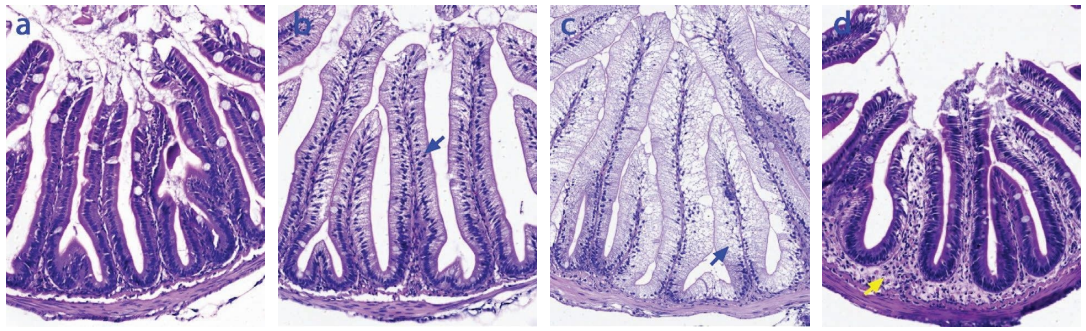

Figure 4: Representative images of pyloric caeca sections. (a) Pyloric caeca mucosa with normal enterocyte morphology alongside, (b) pyloric caeca with marked, (c) severe enterocyte steatosis (blue arrows), and (d) moderate inflammatory change characterized by oedema and an infiltration of the submucosa (yellow arrow) and lamina propria by inflammatory cells.

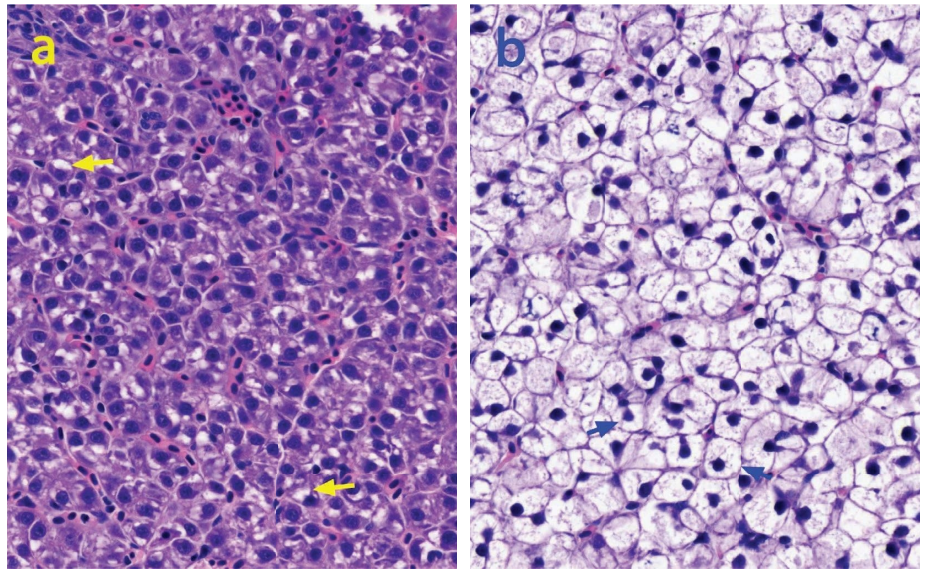

Figure 5: Representative images of the liver sections that were scored as (a) moderate or (b) severe vacuolization of the hepatocytes. The yellow arrows show lipid vacuoles occupying about 50% of the hepatocyte with the nucleus pushed to the side. The blue arrows in image (b) show hepatocytes filled with vacuoles with the nuclei now centrally located in the cell and shrunken.

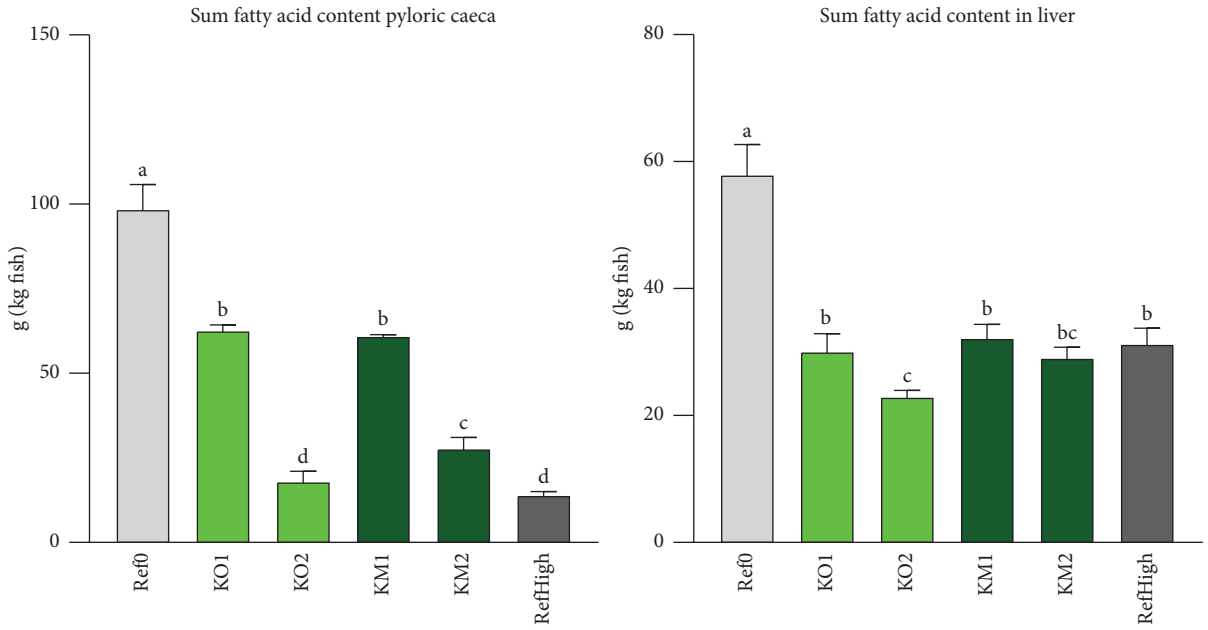

Figure 6: The total fatty acid content in pyloric caeca and liver tissues of fish after 8 weeks of feeding on respective test diets. The fat levels were significantly lower in choline-supplemented diets, with the lowest levels being observed in KO2 and Ref high for pyloric caeca, and in KM2, KO2, and Ref high for liver fat, respectively.

Steatosis was not eliminated even in the Ref High diet in the present study, in which the choline level (3970 mg/kg) was higher than the required (3400 mg/kg) levels as suggested by Hansen et al. [9]. The fact that symptoms of steatosis, although mild, were observed in fish fed with higher levels of choline in the Ref High diet could indicate that the choline requirement of the fish in the present study was higher than of the fish in Hansen et al.'s study. Other possible causes of the apparent differences in the choline requirement could be related to differences in abiotic as well as biotic aspects. Environmental factors such as temperature, 13.8°C versus 10.5°C, and freshwater in the present study vs. seawater in Hansen et al.'s study, respectively, were different. In addition, the lipid level of the diets, 22 vs. 29%, and fish size, 62 g vs. 456 g were different between the present study and in Hansen et al.'s study, respectively. These differences could have led to a difference in the feed intake and growth between the present study and [9], which, could have led to differences in the amount of fat transport in the intestine and eventually the choline needed for growth and lipid transport.

Interestingly, an inverse relationship was observed between the choline supplementation and the liver lipid levels. However, it was not observed for the histological appearance. This could indicate that the vacuoles observed in the liver contained other components than lipid or possibly in addition to lipid. One plausible explanation could be that the majority of the hepatic vacuoles contained glycogen. This is a common phenomenon observed especially in smaller fish, where a higher glycogen concentration in the liver has been shown to be positively associated with growth in Atlantic salmon fry [25]. However, further investigation on the type of vacuoles, e.g., with glycogen specific staining such as periodic acid-Schiff (PAS), would have confirmed this possibility. Unfortunately, the present study could not validate it.

Finally, the authors suggest studies with higher doses of KM and KO to cover a wider range of PC dietary inclusion levels, in order to optimize the choline levels needed to fully eliminate steatosis. Besides, more studies are warranted with different doses of PC from KM and KO for different developmental stages of Atlantic salmon fed different lipid levels.

5. Conclusion

The study showed high and similar ability of choline from KO and KM regarding the reduction of steatosis in the pyloric caeca and a growth-stimulating effect in comparison to highly choline deficient diets. However, further studies are needed with higher choline doses to be able to optimize dietary choline levels for the prevention of steatosis.

Data Availability

The data are mainly presented in the figures and tables of the manuscript submitted. However, if there is a need for raw data, the authors would provide that upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

The study was funded by AkerBioMarine Antarctic AS.

Supplementary Materials

Supplementary Table S1 provides the fatty acid composition of the experimental diets as analyzed, g/kg. (*Supplementary Materials*)

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