Bioavailability of docosahexaenoic acid 22:6(n-3) from enantiopure triacylglycerols and their regioisomeric counterpart in rats

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KEYWORDS

Absorption; Docosahexaenoic acid; (n-3) PUFAs; Plasma and fecal lipids; Stearic acid; Enantiospecificity; Regiospecificity; Structured lipids.

ABBREVIATIONS

TAGs, triacylglycerols; DHA, docosahexaenoic acid; LC (n-3) PUFA, long chain (n-3) polyunsaturated fatty acids; CAL, *Candida antarctica* lipase; *sn*-1 DHA, 2,3-distearoyl-1-docosahexaenoyl-*sn*-glycerol; *sn*-2 DHA, 1,3-distearoyl-2-docosahexaenoyl-*sn*-glycerol; *sn*-3 DHA, 1,2-distearoyl-3-

docosahexaenoyl-*sn*-glycerol; TS, tristearin; NF, standard AIN-93G fed group; GC, gas chromatography; PL, phospholipid; FAME, fatty acid methyl ester; ANOVA, Analysis of Variance; MUFA, monounsaturated fatty acid; SFA, saturated fatty acid; ARA, arachidonic acid; ALA, α-linolenic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; SD, standard deviation.

ABSTRACT

Lack of synthetic enantiospecific triacylglycerols (TAGs) has hindered our understanding of the impact of TAG structure on the absorption and metabolic fate of fatty acids (FAs). In a five-day feeding trial with mildly (n-3) deficient rats, the bioavailability of docosahexaenoic acid [22:6(n-3), DHA] and stearic acid (18:0) from the two different enantiomers of TAG: *sn*-22:6(n-3)-18:0-18:0 and *sn*-18:0-18:0-22:6(n-3), and their regioisomeric TAG: *sn*-18:0-22:6(n-3)-18:0 was compared. Less secretion of fecal DHA was detected from the *sn*-2 position compared with the *sn*-1 and *sn*-3 positions, but no difference was found in DHA content of the fasting plasma or in the weight of the body or organs. 18:0 was lost to feces mainly as cleaved from the primary positions but also as glycerol-bound. The 5-day intervention in rats was long enough to modify the fatty acid profile of plasma phospholipids in rats.

1. INTRODUCTION

Triacylglycerols (TAGs) are to a large extent chiral (Gunstone, Harwood, & Dijkstra, 2007) and they are metabolized in the chiral physiological environment (Carrière et al., 1997; Lehner & Kuksis, 1993; Lehner, Kuksis, & Itabashi, 1993). For humans, dietary TAGs are the source of the essential fatty acids (FAs), linoleic [LA, 18:2(n-6)] and α-linolenic acids [ALA, 18:3(n-3)], as well as of long chain (n-3) polyunsaturated FAs [LC (n-3) PUFA], most importantly docosahexaenoic acid DHA [22:6(n-3)].

In most regions of the world the levels of DHA in the blood of humans are lower than optimal (Stark, Van Elswyk, Higgins, Weatherford, & Salem, 2016). This is alarming as LC (n-3) PUFAs take part in numerous biological functions such as retinal and neural development and prevention of cardiac and circulatory disorders (Hull, 2011; Swanson, Block, & Mousa, 2012). To take part in the biological functions the FAs need to be absorbed. Structures of lipid molecules play an important role in the absorption and metabolism of n-3 PUFAs. N-3 PUFAs fed as alkyl esters and those as TAGs will be absorbed via different routes and will be used for biosynthesis of TAGs by different pathways after absorption (Yang, Kuksis, & Myher, 1990, 1995). However, little is currently known of the relationship between the molecular structures of lipids, especially of the different enantiospecific forms of TAGs, and absorption or metabolic fate of (n-3) FAs *in vivo* (Ghasemifard, Turchini, & Sinclair, 2014). This gap in knowledge has been caused by the challenging synthesis of enantiospecifically structured TAGs in quantities required for animal studies and for clinical investigations in humans.

Hydrolysis of TAGs is performed by lipases, which are typically enantioselective. In the digestive tract, FAs in the *sn*-1 and *sn*-3 positions are cleaved while the FAs of the *sn*-2 position are mainly absorbed with the glycerol backbone as monoacylglycerols (MAG). However, the digestion of the FAs in the *sn*-1 and *sn*-3 positions is not equal. There are indications that lingual and gastric lipases hydrolyze *sn*-3 positon FAs faster than *sn*-1 position FAs as reviewed previously (Duan, 2000; Hamosh, 1984). The pancreatic lipase of the dog has shown enantiopreference for the *sn*-3 position of TAGs and enantiopreference for the *sn*-1 position of racemic diacylglycerols (Carrière et al., 1997). Pancreatic lipase has shown low activity towards (n-3) FAs, in particular over DHA, if located in the *sn*-3 position

of TAG (Bottino, Vandenburg, & Reiser, 1967). The mono- and diacylglycerol transferases in the intestine are known to possess enantiospecificity, and to favor the formation of *sn*-1,2-diacylglycerols over *sn*-2,3-diacylglycerols (Lehner et al., 1993). However, the formed *sn*-1,2- and *sn*-2,3- are utilized at similar rates (Lehner & Kuksis, 1993). Evidence from comparison between fish oil (DHA primarily in *sn*-2 position) and seal oil (DHA primarily in *sn*-1/3 positions) indicates that the digestion, absorption, and metabolism of DHA may be more efficient from the *sn*-2 position compared with the *sn*-1 and *sn*-3 positions of TAGs (M. S. Christensen, Høy, Becker, & Redgrave, 1995). The difference has been seen in the assimilation of DHA in the liver and the brain (Bandarra et al., 2016) as well as in the brown adipose tissue (Lopes et al., 2017). However, the difference has not been validated with TAGs with enantiospecific positioning of DHA. Currently, very little is known of possible differences in the absorption or metabolic fate of DHA between *sn*-1 and *sn*-3 positions.

In pharmacology, the term bioavailability refers to the measurement of the rate and extent to which a drug reaches the systemic circulation (Kwan, 1997). For nutrients, especially for (n-3) FAs, the term is used broadly most often to describe how much of a given nutrient administered orally is retained in the body after digestion and absorption (Ghasemifard et al., 2014). In this study, bioavailability is determined from the assessment of fecal and plasma FA compositions.

The present study was conducted to determine if dietary TAGs possessing DHA either in *sn*-1, *sn*-2 or *sn*-3 position and two stearic acid residues in the remaining *sn*-positions, [*sn*-22:6(n-3)-18:0-18:0, *sn*-18:0-22:6(n-3)-18:0 or *sn*-18:0-18:0-22:6(n-3)], i) would lead to different lipid content and composition of the feces ii) would lead to difference in FA content or composition of fasting plasma or iii) would have a different impact on the weights of the organs. The study was conducted in mildly (n-3) FA deficient rats, and by using both tristearin and normal feed as controls. The study was enabled by chemoenzymatic synthesis of enantiospecific TAGs in quantities sufficient for rat feeding (Halldorsson, Magnusson, & Haraldsson, 2003; Haraldsson, Halldorsson, & Kulås, 2000; Kristinsson, Linderborg, Kallio, & Haraldsson, 2014) and by development of a powerful method of chiral liquid chromatographic analysis for enantiospecific resolution of enantiomers of TAGs necessary for determination of the purity level of the synthesized molecules (Kalpio et al., 2015). To our knowledge, this is the first study

assessing the bioavailability and metabolic fate of DHA from the *sn*-1, *sn*-2 and *sn*-3 positions of TAGs with a feeding period of several days.

2. EXPERIMENTAL SECTION

2.1 Ethics approval

The protocol of the animal experiment was approved by the Medical Ethics Research Board of the Peking University Health Science Center, China (LA2016043).

2.2 Synthesis of regio- and enantiopure structured triacylglycerols

The enantiopure structured TAGs possessing DHA located in the *sn*-1 or *sn*-3 positions along with stearic acid occupying the remaining positions were synthesized in five steps starting from enantiopure (*R*)- and (*S*)-solketals, respectively, as chiral precursors, as reported previously (Kristinsson et al., 2014). Synthesis of the symmetrically structured TAG possessing DHA in the *sn*-2 position and stearic acid in the remaining *sn*-1,3 positions was based on a previously described two-step chemoenzymatic route from glycerol using a highly regioselective immobilized *Candida antarctica* lipase (CAL-B) from Novozymes (Bagsværd, Denmark) (Halldorsson et al., 2003). DHA (≥ 95%) was obtained as ethyl ester from Pronova Biocare (Sandefjord, Norway) and converted into free acid by a previously described method (Haraldsson et al., 2000). All products and intermediates were obtained in excellent chemical and regioisomeric purity (> 98%) as was established by ^{1}H (400 MHz) and ^{13}C NMR and IR spectroscopy as well as satisfactory high-resolution accurate mass spectrometry analyses. Starting materials and products were investigated by 400 MHz ^{1}H NMR to ensure that lipid oxidation did not take place. Excellent enantiopurity (> 96% enantiomeric excess) of the chiral TAGs was fully established by chiral HPLC measurements based on the use of two chiral columns connected to a sample recycling system (Kalpio et al., 2015). The synthesis and the enantiopurity measurements will be reported separately (Magnusson *et al.*, unpublished research). Tristearin was prepared by following a previously reported procedure by treating glycerol with stearic acid by the aid of the immobilized CAL-B under vacuum at 70-75 °C (Haraldsson et al., 2000). All chemical transformations were performed under strict inert atmosphere (nitrogen or argon). The TAGs were stored under nitrogen at -85 °C.

2.3 Animals and diets

Sixty male Sprague-Dawley rats (age 21±2 days) were kept for 7 days in isolation with constant temperature and humidity and on adaptive feeding of standard AIN-93G diet (Table 1) which contained soy bean oil as a source of (n-3) FA (Reeves, Nielsen, & Fahey, 1993) (Nuoyuan biotechnology Co., Ltd, Beijing, China). After the 7 days, the rats were randomly divided into 5 experimental groups of 12 animals each. During an induction phase of four weeks, four groups were fed with AIN-93G containing (n-3) FA deficient peanut oil as the only source of FAs (Table 1) to induce a mild (n-3) FA deficient state, while the fifth group was on the standard AIN-93G containing soy bean oil (NF). During this induction phase, four rats were housed in one cage and their food intake was monitored. After the induction phase, the four (n-3) deficient groups received 2,3-distearoyl-1-docosahexaenoyl-*sn*-glycerol (*sn*-1 DHA), 1,3-distearoyl-2-docosahexaenoyl-*sn*-glycerol (*sn*-2 DHA), or 1,2-distearoyl-3-docosahexaenoyl-*sn*-glycerol (*sn*-3 DHA), or tristearin (TS) as the experimental fat for five days (the intervention phase), whereas the NF group continued on standard AIN-93G diet. α- Tocopherol (100 mg/100 g) was added to the experimental fats before dividing the fat in to individual doses. Individual doses were stored under nitrogen at -80 °C. Based on previously applied doses (Ghasemifard et al., 2014; Kaur et al., 2010), each rat was given a daily dosage of 360 mg of experimental fat embedded between two halves of a low omega-3 FA feed pellet, which served as the first morning feed. Pellets containing the experimental fats were prepared the day before the feeding. The aliquots of experimental fat were melted quickly using a water bath at 40 °C, and pipetted between two halves of feed pellets. The experimental pellets were stored at dark +4 °C overnight and fed to the rats in the morning. Once verified that the experimental fat pellets were consumed completely, the rats were provided with the remainder of the normal daily ration. The NF group was provided with the daily ration only. The rats were provided feed *ad libitum*. The rats were housed individually from the last day of the induction phase until sacrifice on the morning of day 6.

2.4 Sample collection

The rats were weighed daily on the last day of the induction phase (baseline) and throughout the 5-day feeding period. Baseline fecal samples were collected the day before starting the intervention phase. Fecal samples were collected daily during the 5-day intervention period. Immediately after collection and weighing, the fecal samples were frozen and stored at −80 °C. The frozen samples were homogenized by using a mortar and a pestle. The homogenized samples from the intervention phase were pooled to form the fecal study samples. On the sixth day while the rats were in the fasting state, the rats were sedated by inhaling isoflurane and sacrificed with exsanguination. Dry tubes without anticoagulant were used to collect blood samples after the femoral artery was cut off. The blood samples were centrifuged, and the supernatant plasma was collected into sterile tubes and frozen at −80 °C. The brain, eyes, liver, heart, testicles, kidneys, lungs, visceral fat, and the rest of the body, including fur, were collected from each rat and weighed.

2.5 Extraction of lipids, isolation of TAGs and phospholipids, and preparation of FA methyl esters

All analyses were performed in duplicate. All solvents used were of HPLC grade. Total lipids were extracted from plasma with a modified Folch method using methanol, chloroform and 0.88% KCl in milli-Q water (Christie, 1989; Folch, Lees, & Sloane Stanley, 1957). An internal standard mixture of triheptadecanoin (for TAG) and dinonadecanoylphosphatidylcholine (for PL) (Larodan Fine Chemicals AB, Malmö, Sweden) was used. TAGs and PLs were isolated from the extracted plasma lipids with solid phase extraction using Sep-Pak Vac 1cc silica cartridges (Waters, Dublin, Ireland) as previously described (Hamilton & Comai, 1988). Fecal lipids were extracted by a modified Folch method (Folch et al., 1957) and by extracting the solid residues twice. Triheptadecanoin (Larodan Fine Chemicals AB, Malmö, Sweden) was used as an internal standard. To analyze the glycerol-bound FAs, plasma TAG and PL fractions were methylated using the sodium methoxide method (Christie, 1982). In short, the lipids were suspended in dry diethyl ether; thereafter methyl acetate and sodium methoxide were added. The reaction was stopped with addition of acetic acid after 5 min of incubation.

The fecal lipids were methylated with two methods in order to differentiate the glycerol bound FAs from the free FAs. Sodium methoxide was used to analyze the glycerol-bound FAs as described in the

previous section for plasma lipids. A parallel set of the fecal lipids were methylated using an acid-catalyzed method, which transformed both free and esterified FAs into FA methyl esters (FAMEs) by reaction with acetyl chloride/methanol for overnight at 50 °C (Christie, 2003).

2.6 Fatty acid analysis

The FAMEs were analyzed with gas chromatograph (Shimadzu GC-2010 equipped with AOC-20i auto injector, flame ionization detector, Shimadzu corporation, Kyoto, Japan) equipped with a wall-coated open tubular column DB-23 (60 m x 0.25 mm i.d., liquid film 0.25 μm, Agilent Technologies, J.W. Scientific, Santa Clara, CA, USA) using helium as the carrier gas. A splitless injection mode (volume 0.5 μL) was used, and the split was opened after 1 min. The temperatures were: inlet 270 ºC; oven 130 ºC held 1 min, 6.5 ºC/min to 170 ºC, 2.75 ºC/min to 200 ºC, held for 21 min, 40 ºC/min to 230 ºC and held for 2 min; detector 280 ºC. The peaks were identified by comparing the retention times with those of external standards [Supelco 37 Component FAME mix (Supelco, St. Louis, MO, USA), 68D (Nu-Check-Prep, Elysian, MN, USA), and GLC-490 (Nu-Check-Prep, Elysian, MN, USA)]. Correction factors were used based on the external standards. Quantification (μg/100 mg of sample) was based on the internal standards. Molar percent of fecal DHA and 18:0 in relation to the fed doses were calculated.

2.7 Statistical analysis

Statistical analyses were performed with the SPSS 23 program (IBM, Armonk, NY, USA) to evaluate the significance of differences of the FA composition of total lipids in rat feces and of plasma TAG and PL, and to determine differences in the rat body and organ weights among the different intervention groups. All the data were checked for normality and variance homogeneity, and reported as means ± standard deviation (SD). Analysis of Variance (ANOVA) was tested for significance between the intervention groups and followed by Tukey's HSD with Bonferroni corrections. Tamhane's T2 analysis was used when variances were not homogenous. Statistical significance was determined at $p < 0.05$. Non-parametric Kruskal-Wallis test followed by Mann-Whitney U test was performed when the data were not normally distributed.

3. RESULTS AND DISCUSSION

3.1 Excluded samples

One rat from the *sn*-1 DHA group was excluded from the body weight analysis on day 5 because of unexplained weight loss. Testis weight of one rat in the *sn*-1 DHA group was excluded because of underdeveloped appearance. Two rats from *sn*-3 DHA group were excluded from plasma lipid analysis since their lipid content was standing out from the rest of the group (more than 2SD away from the mean, Supplementary table S5).

3.2 Fecal lipids

Fecal FA composition (Supplementary tables S1-S4) reflected the FA composition of the feed (Table 2). Peanut oil caused more fecal lipid loss than soybean oil (Table 3). Largest contributors to the difference were the 20:0, 22:0 and 24:0 as well as oleic acid. In peanut oil, SFAs are located predominantly in the *sn*-3 position (Myher, Marai, & Kuksis, 1977; Hiromi Yoshida, Hirakawa, Tomiyama, Nagamizu, & Mizushina, 2005) while in soybean oil, SFA primarily occupy the *sn*-1/3 positions (Takagi & Ando, 1991; Hiromi Yoshida, Kanei, Tomiyama, & Mizushina, 2006). In both peanut and soy bean oil oleic acid is evenly distributed within the three *sn* -positions. The level of ALA was significantly higher in the NF groups than in other groups.

Even though supplementation of DHA increased its content in the feces in all DHA fed groups compared with the non-DHA fed groups (Table 3), the fecal DHA represented 0.1-0.5% (molar percent) of total DHA fed to the rats (Figure 1C), which indicates an overall high extent of absorption of DHA regardless of the positional distribution of DHA in TAGs. The total concentration as well as the molar percentage of DHA in the feces were four times higher and that of the glycerol-bound DHA more than five times higher in the *sn*-1 DHA and *sn*-3 DHA groups compared with the corresponding levels in the *sn*-2 DHA group (Table 3, Figure 1C), indicating superior extent of absorption of DHA from the *sn*-2 position compared with *sn*-1 and *sn*-3 positions. The total fecal DHA level was 2-3 times higher than the glycerol-bound DHA (Table 3), which suggests that a significant part of the DHA was excreted as free FA. Chylomicron TAGs of rats fed oil have retained about 85% of the original fatty acids in the

233 *sn*-2 position (Yang & Kuksis, 1991). Thus, interesterification of *sn*-2 monoacylglycerol to *sn*-1/3
234 monoacylglycerol and subsequent hydrolysis during digestion may explain the presence of non-glycerol
235 bound DHA in the feces of the *sn*-2 DHA group (Couëdelo et al., 2012; Karupaiah & Sundram, 2007).
236 Differences between the fecal loss of DHA from *sn*-1 and *sn*-3 positions were not detected (Table 3).
237 Higher content of docosapentaenoic acid [22:5(n-3), DPA] was detected in *sn*-1 and *sn*-3 DHA groups
238 than in the *sn*-2 group, which could indicate retroconversion from DHA to DPA in the digestive tract
239 (Supplementary table S1).

240 Stearic acid cleaved from the *sn*-1 and *sn*-3 positions was lost to feces to greater extent than stearic acid
241 of the *sn*-2 position ($p = 0.001$, $p = 0.004$ respectively, Table 3, Figure 1B). The loss of SFA's to feces
242 as calcium and magnesium soaps has been described previously (Brink, Haddeman, de Fouw, &
243 Weststrate, 1995; Jandacek, 1991; Mattson, Nolen, & Webb, 1979). Overall, in the *sn*-1 DHA, *sn*-2
244 DHA and *sn*-3 DHA groups the fecal fat loss in the glycerol-bound FA forms was only about 5-7% of
245 the total lipid loss (Table 3). Within that, the loss of the glycerol bound 18:0 ($p < 0.01$) was lower in
246 the *sn*-2 DHA group compared with the *sn*-1 DHA and *sn*-3 DHA groups (Table 3, Figure 1A), which
247 can indicate low absorption of distearin.

248 Saturated FAs were included in the experimental TAGs due to the design of the synthesis. Stearic acid
249 was chosen over palmitic or myristic acids, because stearic acid is less hypercholesterolemic possibly
250 because of its rapid conversion to oleic acid (Bruce & Salter, 1996). TS doubled the amount of lipids
251 lost to feces when compared with in the *sn*-1 DHA, *sn*-2 DHA and *sn*-3 DHA containing feed. Lipid
252 loss in the TS group was fourfold when compared with NF group (Table 3). Based on the average feed
253 consumption measured during the introductory period, peanut oil feed provided about 0.05 g and soy
254 bean oil about 0.07 g of stearic acid daily. This corresponds to about 22% of stearic acid fed in during
255 the experimental period in the DHA fed groups and 15% in the TS group. By a rough estimation not
256 taking into account the stearic acid in the feed or endogenous stearic acid, about 17 molar percent of
257 fed stearic acid was lost to feces in the tristearin group (Figure 1D). Corresponding loss was about 10%
258 in the *sn*-1 DHA and *sn*-3 DHA groups and about 12% in the *sn*-2 DHA group. The loss of stearic acid
259 esterified with glycerol was less than 0.4% molar percent in the *sn*-1 DHA, *sn*-2 DHA and *sn*-3 DHA

groups, while most (15% vs. 17%) of the total loss of stearic acid in the TS group was bound in glycerol. While the reduced lipolysis as well as reduced transportation to lymph of tristearin compared with triolein has been detected previously (Bergstedt, Hayashi, Kritchevsky, & Tso, 1990), the absorption has been shown to increase when other lipids are ingested together with tristearin (Bergstedt et al., 1991).

3.3 Plasma lipids

The fasting plasma contained more than double the amount of PLs compared with TAGs (Tables 4A and 4B). No differences were found in the total amount of TAGs in the fasting plasma between the groups, but plasma of the NF group had more PLs than the TS group ($p = 0.03$).

Feeding DHA increased the DHA content in the plasma TAGs and PLs compared to the TS or NF groups (Tables 4A and 4B). No significant differences were found between the *sn*-1 DHA, *sn*-2 DHA and *sn*-3 DHA groups in the levels of DHA, stearic acid or any other FAs in the fasting plasma TAGs or PLs. Due to the overall high efficiency of DHA absorption in all of the DHA fed groups, the differences in the fecal loss of DHA between the groups fed with DHA groups were not reflected to fasting plasma. In contrast, Yoshida et al. (1996) found that *sn*-1/3 DHA dominating seal oil caused lower TAG concentrations in rat plasma compared with *sn*-2 DHA dominating fish oil. However, their feeding was significantly longer (160 days), and the fed FA composition different from ours. In a study with mice, the positional distribution of EPA, but not DHA, affected the incorporation of these FAs into monocytes and neutrophils (Kew et al., 2003).

The composition of plasma FAs reflected the FA profile of the feed, which is seen for example in the contents of oleic acid and LA (Tables 4A and 4B). The DHA level was higher in the NF group compared with the TS group in both TAGs and PLs ($p = 0.008$, $p < 0.01$ respectively), which indicates conversion of ALA to DHA in the NF fed group (Table 2). The DHA levels in plasma TAGs ($p = 0.008$, $p = 0.001$, $p = 0.001$ respectively) and PLs group ($p = 0.07$, $p < 0.01$, $p = 0.04$ respectively, significant difference to *sn*-2 and *sn*-3 groups) of rats fed with DHA compared to the plasma DHA status of NF rats indicate

that the DHA dose in our study was at least adequate, if not high. The results also showed that the 5-day intervention was long enough to modify the FA profile of plasma phospholipids in rats.

Retroconversion of DHA to eicosapentaenoic acid [EPA, 20:5(n-3)] in the DHA fed groups and conversion of ALA to EPA in the NF group caused the EPA content to be equal in the DHA fed groups and NF group and higher than in the TS group in plasma TAGs ($p < 0.05$). Similar trend was seen in the plasma PLs (Table 4B). Higher LA content in the feed (Table 2) caused the higher content of arachidonic acid [ARA, 20:4(n-6)] in plasma PLs of the NF group compared with the other groups. In plasma TAGs, the content of ARA in the TS and NF groups was equal and higher than ARA levels in the DHA fed groups (Tables 4A). The level of ARA in plasma PLs was significantly higher in TS group than in the *sn*-3 DHA group ($p < 0.05$, Table 4B). This indicates compensation of the lack of DHA with ARA as detected previously (Wainwright et al., 1999).

Our study supports previous findings on the absorption of (n-3) PUFA located in the *sn*-2 position being more efficient compared with the (n-3) PUFA located in the *sn*-1/3 positions (Linderborg & Kallio, 2005; Mu & Porsgaard, 2005). The absorption of DHA to lymph has been shown to be more efficient from the *sn*-2 position compared with the randomized positions from TAGs with two decanoic acid residues and one DHA residue in rats (M. S. Christensen et al., 1995). Further, the absorption of DHA has been shown to be faster from fish oil (DHA predominantly in the *sn*-2 position) than from seal oil (DHA predominantly in the *sn*-1/3 positions) (Ikeda et al., 1995). However, with longer lymph collection times (24 hours) the total amount of the FAs absorbed from fish oil and seal oil in 24 hours has not shown a difference (M. S. Christensen, Høy, & Redgrave, 1994). Previously, feeding natural fats differing in the positional distribution of PUFAs for 24 days has not caused significant differences in the lipid metabolism of rats (De Schrijver, Vermeulen, & Viaene, 1991) nor has the feeding of fish oil or seal oil for 17 days caused different FA profiles in rat tissue PLs (Christensen & Høy, 1992).

3.4 Body and organ weights

At baseline or during the intervention days 1-4 there were no significant differences in the body weights of the rats (Table 5A) possibly due to the *ad libitum* availability of feed. The NF group was the only

group that gained weight during the 5-day intervention feeding and on day 5, the NF group had the highest body weight (statistically significant difference to *sn*-1 group only, $p = 0.014$). This may indicate some effect of the fecal fat loss which was lowest in the NF group and highest in the TS group or adaptation to the few experimental pellets prior to their daily ration. Despite the relatively short period of experimental feeding, the effect of lower weight gain in the DHA groups may have resulted from the (n-3) PUFA's anti-obesity properties (Li, Huang, & Xie, 2008). Such properties have been seen in rodent models by Hassanali, Ametaj, Field, Proctor, & Vine, (2010) and Li et al. (2017) but not by Bandarra et al. (2016). The weight of the livers in the NF group was significantly higher compared with livers of the other groups, but the liver to body weight ratios did not differ significantly (data not shown). The fresh weight of eyes, brain, lungs, heart, testicles, kidneys or visceral fat did not differ between the groups (Table 5B).

3.5 Strengths and limitations

Our study has several strengths. Synthetic enantiospecific structured TAGs enabled the direct comparison of the positional distribution of DHA and stearic acid without the interference of other FAs which has been a problem in most previous studies using interesterified mixtures or natural oils. Inclusion of the two control groups, TS and NF, enabled assessment of the (n-3) FA deficiency status and the status of our experimental groups to the typically fed laboratory animals. Analysis of the fecal fats by two different methods enhances understanding of the mechanisms behind the fecal fat loss. In previous studies, the analysis of fecal lipids has been largely omitted from the bioavailability trials of LC (n-3) PUFA, even to the extent that a recent review sees the fecal analysis simply as a missed opportunity (Ghasemifard et al., 2014) to achieve robust and informative results about the efficacy of the different LC (n-3) PUFA preparations. Limitations include that our setting did not take into account the possible effect of gut microbiota on the fate of the FAs which were not absorbed in the small intestine and entered the colon. However, the research group is not aware of data available on whether certain gut microflora would use DHA or stearic acid more or less efficiently than another type, nor is there any research on the effect of TAG structure on such metabolism. For the two rats with larger livers, although there was no significant difference of liver to body weight ratios between the groups,

no pathological analysis on livers was carried out. Another challenge lies in the estimation of optimal baseline (n-3) status, dose and length of intervention for detection of minor differences, if any, between the *sn*-1 and *sn*-3 positions. A fourth challenge lies in extrapolation of the results obtained in rats to humans. Anatomical and physiological differences exist between the gastro-intestinal tract of rats and humans as reviewed by DeSesso & Jacobson (2001), and differences, e.g. in bile acid concentrations, can affect the digestion and absoption of SFAs.

4. CONCLUDING REMARKS

Information available on the lipid-structure related to bioavailability of LC (n-3) PUFA, especially of DHA, is currently scarce (Ghasemifard et al., 2014; Lopes et al., 2017). To our understanding, this is the first study reporting findings of the bioavailability of DHA from enantiospecific TAGs in an animal model. We substantiated superior bioavailability of DHA from the *sn*-2 position compared with the *sn*-1 and *sn*-3 positions as measured by the amount of DHA lost to feces, but did not substantiate differences between the two primary positions (*sn*-1 and *sn*-3). Our study supports earlier findings on the loss of stearic acid to feces especially when located in the *sn*-1 and *sn*-3 positions.

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AUTHORS CONTRIBUTIONS

The work was initially designed by KL, BY, GH and YZ. Synthesis of the enantiopure TAGs was conducted by GH and JM. Animal study was conducted by AZ and JZ. AK performed the extractions, isolations, GC analysis as well as data collection and data analysis. The manuscript was drafted by KL and AK, and it was edited and approved by all authors.

CONFLICT OF INTEREST

All authors declare that they have no conflict of interest.

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535 Table 1: Composition of diets.

Ingredients	Contents (g/kg)
Corn starch	397
Casein (>85% protein)	200
Corn dextrin	132
Sucrose	100
Oil[1]	70
Fiber	50
Minerals	35.5
Vitamins	10
L-cystinol	3
Choline bitartrate	2.5
Tert-butylhydroquinone	0.014

536 [1] Low omega-3 FA diet (modified AIN-93G diet) for *sn*-1 DHA, *sn*-2 DHA, *sn*-3 DHA and TS groups
537 contained peanut oil. Standard omega-3 AIN-93G diet for NF group contained soy bean oil.

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548 Table 2. Fatty acid composition of oils used in the feed. Values are expressed as mean mass percentages

549 of two replicates.

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Fatty acid	Peanut oil[1]	Soy bean oil[2]
16:0	9.7	10.8
18:0	3.2	4.4
18:1(n-9)	61.6	22.7
18:2(n-6)	17.9	51.9
18:3(n-3)	0.1	6.3
20:0	1.4	0.4
22:0	2.5	0.4
24:0	1.3	0.1
Others[3]	2.4	3.0

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552 [1] Peanut oil was used in the feed of the *sn*-1 DHA, *sn*-2 DHA, *sn*-3 DHA and tristearin (TS) groups

553 [2] Soy bean oil was used in the feed of the normal feed (NF) group

554 [3] This category includes 16:1(n-7), 18:1(n-7), 18:3(n-6), 20:1(n-9) and 23:0.

555 Table 3. Total lipid and DHA content excreted in feces (µg/100 mg) of different intervention groups at the baseline and during the five-day intervention feeding
556 phase.

Lipid	Groups[1]				
	sn-1 DHA	*sn*-2 DHA	*sn*-3 DHA	Tristearin	Normal feed
	Glycerol-bound fatty acids				
DHA in study feces[2]	8.58±3.14^{b}	1.69±0.42^{c}	10.72±2.37^{b}	0.20±0.17^{a}	0.17±0.04^{a}
DHA in baseline feces[3]	0.12±0.04^{b}	0.14±0.09^{ab}	0.14±0.05^{b}	0.12±0.04^{b}	0.22±0.05^{a}
Total lipids in study feces[4]	135.2±35.4^{ac}	121.6±19.3^{a}	149.8±23.1^{c}	2541.1±204.8^{b}	127.5±21.5^{ac}
Total lipids in baseline feces[5]	135.7±18.5^{ab}	142.74±17.0^{a}	125.8±13.7^{ab}	121.7±13.5^{b}	126.8±18.4^{ab}
	Total fatty acids				
DHA in study feces[2]	22.41±5.55^{b}	4.82±1.82^{c}	21.08±7.23^{b}	0.62±0.21^{a}	0.77±0.26^{a}
DHA in baseline feces[3]	0.20±0.14^{ab}	0.22±0.15^{ab}	0.27±0.15^{ab}	0.16±0.1^{b}	0.36±0.14^{a}
Total lipids in study feces[4]	2056.4±232.4^{bc}	2272.2±144.2^{b}	1972.3±239.2^{c}	3904.9±332.4^{a}	1098.0±196.9^{d}
Total lipids in baseline feces[5]	1669.0±183.3^{ab}	1951.1±314.5^{a}	1675.7±411.2^{ab}	1577.0±234.5^{b}	1110.2±244.7^{c}

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558 [1] The groups *sn*-1 DHA, *sn*-2 DHA and *sn*-3 DHA and tristearin received omega-3 FA deficient feed for the 4-week induction period. Thereafter, for the 5 day
559 intervention period the *sn*-1 DHA, *sn*-2 DHA, *sn*-3 DHA groups received structured triacylglycerols with DHA in the indicated *sn*-position and two stearic acid
560 residues in the other positions (360 mg/day), and the tristearin group received tristearin for the 5 day intervention period, in addition to the *ad libitum* omega-3
561 FA deficient feed. During the 4-week induction period and the 5 day intervention period the normal feed group received soy bean oil based normal feed.

562 [2] DHA content in fecal samples from the 5 days of intervention feeding; [3] DHA content in fecal samples collected on last day of induction phase; [4]Average total
563 lipid content in the feces collected during the 5 days of intervention feeding phase; [5] Average total lipid content in the feces collected on the last day of induction
564 phase.

565 Values are mean ± SD, n=12 in each group. Values with different letters in same row differ significantly ($p < 0.05$).

566 Table 4A. Fatty acids of the TAG fractions of fasting rat plasma (μg/100 mg) of different intervention

567 groups

	Groups[1]				
Fatty acids	*sn*-1 DHA	*sn*-2 DHA	*sn*-3 DHA	Tristearin	Normal feed
14:0	0.28±0.15	0.28±0.13	0.37±0.28	0.27±0.11	0.46±0.40
16:0	9.56±4.39	9.92±4.19	8.70±2.41	9.76±3.33	7.56±3.39
16:1(n-7)	0.65±0.37	0.76±0.44	0.63±0.22	0.78±0.31	1.0±0.58
18:0	2.70±1.03	2.74±0.8	2.48±0.87	3.01±1.08	2.35±1.05
18:1(n-9)	14.2±6.6^{ab}	14.2±5.6^{ab}	11.7±3.2^{ab}	15.1±4.9^{a}	8.3±3.9^{b}
18:1(n-7)	0.78±0.41	0.93±0.38	0.78±0.25	0.93±0.30	1.04±0.48
18:2(n-6)	6.16±2.66^{b}	6.36±2.33^{ab}	5.49±1.35^{b}	5.88±1.91^{b}	11.54±5.06^{a}
18:3(n-6)	0.16±0.15^{ab}	0.1±0.03^{b}	0.09±0.02^{b}	0.19±0.07^{a}	0.23±0.10^{a}
18:3(n-3)	0.12±0.07^{a}	0.14±0.05^{a}	0.11±0.03^{a}	0.10±0.05^{a}	0.91±0.42^{b}
20:0	0.17±0.10	0.14±0.04	0.12±0.04	0.14±0.06	0.13±0.02
20:1(n-9)	0.45±0.49	0.37±0.20	0.38±0.30	0.40±0.16	0.31±0.18
20:2(n-6)	0.35±0.16^{a}	0.29±0.10^{a}	0.25±0.10^{a}	0.53±0.16^{b}	0.21±0.11^{a}
20:4(n-6)	3.28±1.10^{ab}	2.95±0.76^{b}	3.0±0.84^{b}	4.57±1.10^{a}	4.42±1.33^{a}
20:5(n-3)	0.43±0.26^{a}	0.52±0.22^{a}	0.37±0.16^{a}	0.07±0.06^{b}	0.47±0.19^{a}
22:0	0.02±0.01	0.02±0.01	0.02±0.02	0.03±0.01	0.01±0.0
22:1(n-9)	0.23±0.28	0.12±0.02	0.17±0.16	0.13±0.03	0.11±0.03
22:4(n-6)	0.14±0.07^{a}	0.13±0.08^{a}	0.12±0.05^{a}	0.45±0.19^{b}	0.14±0.12^{a}
22:5(n-3)	0.02±0.02	0.03±0.01	0.03±0.03	0.03±0.01	0.02±0.01
24:0	0.11±0.07^{ab}	0.13±0.08^{a}	0.12±0.05^{a}	0.04±0.02^{b}	0.21±0.16^{a}
22:6(n-3)	3.58±1.91^{a}	3.46±1.40^{a}	2.95±0.92^{a}	0.29±0.10^{b}	1.06±0.58^{c}
Total SFA	**12.9±5.4**	**13.3±4.7**	**11.8±2.9**	**13.3±4.1**	**10.8±4.4**
Total MUFA	**16.3±7.2**	**16.4±6.4**	**13.7±3.8**	**17.3±5.4**	**10.7±5.1**
Total (n-3) FA	**4.2±2.2^{b}**	**4.3±1.6^{b}**	**3.6±1.1^{b}**	**0.7±0.3^{a}**	**2.7±1.2^{b}**
Total (n-6) FA	**10.1±3.7^{ab}**	**9.9±3.1^{b}**	**8.9±2.27^{b}**	**11.6±3.2^{ab}**	**16.6±6.1^{a}**
Total PUFA	**14.3±5.7**	**14.0±4.7**	**12.4±3.1**	**12.2±3.4**	**19.0±7.2**
Total TAG	**43.5±17.7**	**43.7±15.6**	**37.9±9.4**	**42.8±12.1**	**40.5±16.5**

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Table 4B. Fatty acids of the PL fractions of fasting rat plasma (µg/100 mg) of different intervention groups

	Groups[1]				
Fatty acids	*sn*-1 DHA	*sn*-2 DHA	*sn*-3 DHA	Tristearin	Normal feed
14:0	0.17±0.05^{b}	0.17±0.04^{b}	0.24±0.09^{ab}	0.19±0.03^{b}	0.31±0.05^{a}
16:0	24.67±4.99^{b}	26.22±4.19^{ab}	26.30±4.93^{ab}	25.82±3.74^{ab}	30.38±4.72^{a}
16:1(n-7)	0.35±0.09^{b}	0.38±0.14^{b}	0.43±0.12^{b}	0.46±0.18^{b}	0.65±0.14^{a}
18:0	25.05±3.59^{ab}	24.63±3.81^{ab}	21.01±3.07^{b}	24.52±4.08^{ab}	26.50±5.19^{a}
18:1(n-9)	7.70±1.64^{b}	7.71±1.38^{b}	7.77±1.73^{b}	8.40±1.52^{b}	4.60±0.85^{a}
18:1(n-7)	1.67±0.41^{b}	1.97±0.43^{b}	1.93±0.44^{b}	2.15±0.53^{b}	4.11±0.95^{a}
18:2(n-6)	9.62±2.09^{b}	9.84±1.17^{b}	9.13±2.15^{b}	8.34±1.47^{b}	16.26±2.89^{a}
18:3(n-6)	0.02±0.01^{b}	0.02±0.01^{b}	0.02±0.01^{b}	0.03±0.01^{b}	0.06±0.01^{a}
18:3(n-3)	0.01±0.01^{b}	0.01±0.0^{b}	0.01±0.0^{b}	0.01±0.0^{b}	0.05±0.01^{a}
20:0	0.24±0.13	0.23±0.13	0.23±0.19	0.23±0.11	0.17±0.12
20:1(n-9)	0.19±0.05	0.18±0.03	0.19±0.04	0.21±0.08	0.19±0.04
20:2(n-6)	0.45±0.16^{b}	0.57±0.17^{ab}	0.51±0.19^{ab}	0.76±0.32^{a}	0.73±0.22^{a}
20:3(n-6)	0.74±0.20^{ab}	0.89±0.25^{a}	0.75±0.19^{ab}	0.51±0.19^{b}	1.0±0.26^{a}
20:4(n-6)	21.84±4.76^{bc}	21.84±40^{bc}	18.68±3.30^{c}	26.0±4.95^{b}	31.38±5.58^{a}
20:5(n-3)	0.11±0.06^{bc}	0.12±0.05^{c}	0.08±0.03^{bc}	0.01±0^{a}	0.07±0.01^{b}
22:0	0.03±0.0	0.03±0.0	0.03±0.0	0.04±0.01	0.03±0.0
22:1(n-9)	0.43±0.18	0.36±0.08	0.36±0.10	0.35±0.10	0.27±0.07
22:4(n-6)	0.36±0.35^{b}	0.27±0.06^{b}	0.26±0.07^{b}	2.3±0.77^{a}	0.43±0.28^{b}
22:5(n-3)	0.04±0.04	0.02±0.0	0.02±0.01	0.02±0.01	0.02±0.0
24:0	0.25±0.08^{b}	0.34±0.17^{b}	0.28±0.08^{b}	0.27±0.08^{b}	0.78±0.17^{a}
22:6(n-3)	9.88±2.16^{ab}	10.69±1.96^{a}	9.81±1.88^{a}	3.71±0.61^{c}	7.26±2.04^{b}
Total SFA	**50.4±7.5**	**51.6±7.0**	**48.1±7.7**	**51.1±7.1**	**58.2±9.8**
Total MUFA	**10.4±2.2**	**10.6±1.9**	**10.7±2.2**	**11.6±2.1**	**9.8±1.8**
Total (n-3) FA	**10.1±2.2^{ac}**	**10.9±2.0^{c}**	**9.9±1.9^{c}**	**3.8±0.6^{b}**	**7.4±2.1^{a}**
Total (n-6) FA	**33.1±6.3^{bc}**	**33.5±5.0^{bc}**	**29.4±5.4^{c}**	**38.0±6.8^{b}**	**49.9±8.2^{a}**
Total PUFA	**43.1±7.8^{b}**	**44.3±6.8^{b}**	**39.3±7.1^{b}**	**41.7±7.2^{b}**	**57.3±9.7^{a}**
Total PL	**104.2±16.9^{ab}**	**106.9±15.2^{ab}**	**98.5±16.3 ^{ab}**	**104.7±16.1^{a}**	**125.7±21.0^{b}**

[1] The groups *sn*-1 DHA, *sn*-2 DHA, *sn*-3 DHA and tristearin received omega-3 FA deficient diet for the 4-week induction period. Thereafter, for the 5 day intervention period the *sn*-1 DHA, *sn*-2 DHA, *sn*-3

DHA groups received structured triacylglycerols with DHA in the indicated *sn*-position and two stearic acid residues in the other positions (360 mg/day for each rat), and the tristearin group received tristearin (360 mg/day for each rat) for the 5 day intervention period, in addition to the *ad libitum* omega-3 FA deficient diet. During the 4-week induction period and the 5 day intervention period the normal feed group received soy bean oil based normal feed.

SFA: saturated fatty acids

MUFA: monounsaturated fatty acids

PUFA: polyunsaturated fatty acids

Values are mean ± SD, n=12 except group *sn*-3 (n=10).Values with different superscripts in same row differ significantly ($p < 0.05$).

598 Table 5A. The body weight (g) of the rats during the last day of the induction phase (baseline) and
599 during the five days of intervention

	Group[1]				
	sn-1 DHA	*sn*-2 DHA	*sn*-3 DHA	Tristearin	Normal feed
Baseline[2]	325±34	336±15	346±27	336±21	326±36
Day 1	310±32	322±13	332±26	321±21	337±36
Day 2	333±34	343±14	352±24	336±22	343±34
Day 3	330±32	338±15	343±25	332±20	348±35
Day 4	342±33	354±13	359±18	348±22	349±35
Day 5[3]	328±32[b]	339±13[ab]	344±22[ab]	333±22[ab]	364±35[a]

600

601 Table 5B. Weight of rat organs (g) after the intervention phase

	Group[1]				
	sn-1 DHA	*sn*-2 DHA	*sn*-3 DHA	Tristearin	Normal feed
Eyes	0.3±0.02	0.3±0.03	0.3±0.02	0.3±0.03	0.3±0.02
Brain	2.0±0.1	2.0±0.07	2.0±0.07	2.0±0.1	2.0±0.2
Liver[3]	9.8±1.2[b]	10.4±1.02[b]	11.0±0.9[b]	10.2±0.9[b]	12.5±1.5[a]
Heart	1.3±0.1	1.3±0.1	1.4±0.2	1.3±0.2	1.3±0.1
Lungs	1.4±0.1	1.4±0.2	1.5±0.12	1.5±0.2	1.5±0.2
Testis	3.0±0.2	3.0±0.2	3.0±0.3	3.0±0.2	3.0±0.3
Kidneys	2.7±0.3	2.7±0.2	2.8±0.3	2.8±0.2	2.8±0.1
Visceral Fat	4.2±2.0	5.1±1.3	4.9±1.5	4.0±1.23	5.2±1.6

602

603 [1] The groups *sn*-1 DHA, *sn*-2 DHA and *sn*-3 DHA and tristearin received omega-3 FA deficient diet
604 for the 4-week induction period. Thereafter, for the 5 day intervention period the *sn*-1 DHA, *sn*-2 DHA,
605 *sn*-3 DHA -groups received structured triacylglycerols with DHA in the indicated *sn*-position and two
606 stearic acid residues in the other positions (360 mg/day), and the tristearin group received tristearin for
607 the 5 day intervention period, in addition to the *ad libitum* omega-3 FA deficient diet. During the 4-
608 week induction period and the 5 day intervention period the normal feed group received soy bean oil
609 based normal feed.

[2] Body weight after the 4-week induction phase on peanut oil (*sn*-1 DHA, *sn*-2 DHA, *sn*-3 DHA and TS) or soy bean oil (NF) based feed.

Values for body weight are mean (g) ± SD, n=12 except group *sn*-1 on day 5 where n=11.

Values for organ weight are mean ± SD, n=12 except testis of *sn*-1 group (n=11).

[3]Values with different superscripts differ significantly in each column ($p < 0.05$).

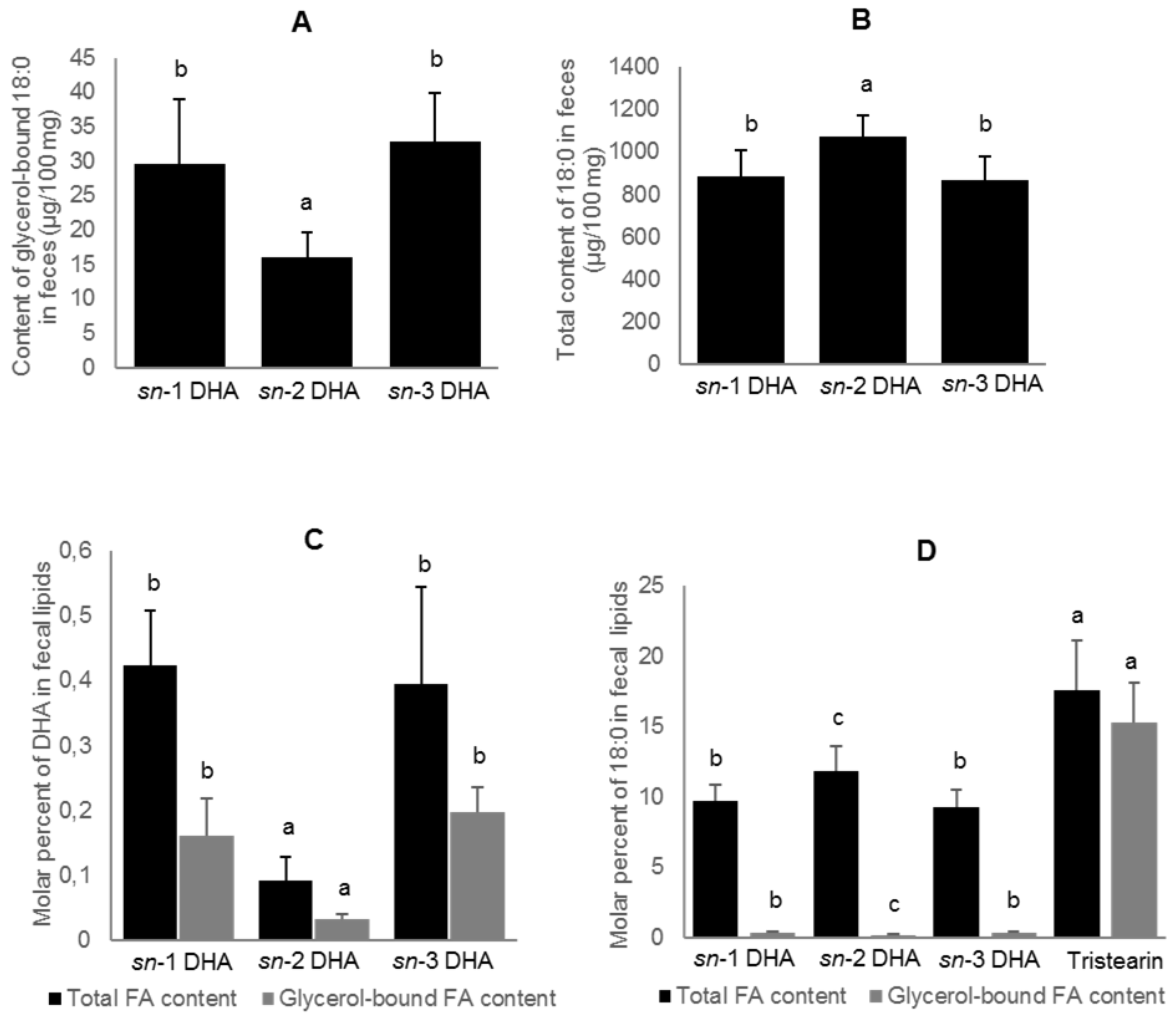

Figure 1. Fecal content of [A] glycerol-bound stearic acid (µg stearic acid in 100 mg feces ± SD) and [B] total stearic acid (µg stearic acid in 100 mg feces ± SD) [C] total and glycerol-bound DHA in molar percent (moles in the feces during the five day intervention period compared with the moles fed in the structured TAGs) (mean ± SD) [D] stearic acid in molar percent (mean ± SD) during the five days of intervention in rats fed with distearoyl-docosahexaenoyl-glycerols with DHA in *sn*-1, *sn*-2 or *sn*-3 positions and tristearin. n=12. Bars with different letters differ from one another within the fatty acid type (total or glycerol-bound).