

Comparison of Triglycerides and Phospholipids as Supplemental Sources of Dietary Long-Chain Polyunsaturated Fatty Acids in Piglets^{1,2}

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ABSTRACT Addition of arachidonic acid (AA) and docosahexaenoic acid (DHA) to infant formula promotes visual and neural development. This study was designed to determine whether the source of dietary long-chain polyunsaturated fatty acids (LCPUFA) affected overall animal health and safety. Piglets consumed ad libitum from 1 to 16 d of age a skim milk-based formula with different fat sources added to provide 50% of the metabolizable energy. Treatment groups were as follows: control (CNTL; no added LCPUFA), egg phospholipid (PL), algal/fungal triglyceride (TG) oils, TG plus PL (soy lecithin source) added to match phospholipid treatment (TG + PL) and essential fatty acid deficient (EFAD). Formulas with LCPUFA provided 0.6 and 0.3 g/100 g total fatty acids as AA and DHA, respectively. CNTL piglets had 40% longer ileal villi than PL piglets ($P < 0.03$), but the TG group was not different from the CNTL group. Gross liver histology did not differ among any of the formula-fed groups ($P > 0.1$). Apparent dry matter digestibility was 10% greater in CNTL, TG and TG + PL groups compared with PL piglets ($P < 0.002$). No differences in alanine aminotransferase were detected among treatments, but aspartate aminotransferase was elevated ($P < 0.03$) in PL piglets compared with TG + PL piglets. Total plasma AA concentration was greater in the TG group compared with CNTL piglets ($P < 0.05$). Total plasma DHA concentrations were greater in TG piglets compared with PL ($P < 0.06$) or CNTL ($P < 0.02$) piglets. These data demonstrate that the algal/fungal TG sources of DHA and AA may be a more appropriate supplement for infant formulas than the egg PL source based on piglet plasma fatty acid profiles and apparent dry matter digestibilities. *J. Nutr.* 132: 3081–3089, 2002.

KEY WORDS: • long-chain polyunsaturated fatty acid • pigs • neonates • arachidonic acid • docosahexaenoic acid

The essential fatty acids (EFA)⁴ linoleic acid [LA; 18:2(*n*-6)] and α -linolenic acid [LN; 18:3(*n*-3)] are necessary for the growth and development of human infants. Proper development of the brain, retina and other body tissues depends upon provision of arachidonic acid (AA) and docosahexaenoic acid (DHA) either directly in the diet or through synthesis from LA and LN (1). The precursors, LA and LN, are primarily in plasma transport or storage lipids in the body, whereas AA and DHA, the EFA metabolites, are major components within the

phospholipid (PL) membrane of cells (2). Intrauterine accretion of AA and DHA occurs largely during the third trimester of pregnancy; therefore, premature infants may be at increased risk for a deficiency (3).

Human breast milk naturally contains LA and LN as well as varying concentrations of preformed AA and DHA, all of which depend on the maternal diet. Concentrations of LA range from 11 to 21 g/100 g total fatty acids and concentrations of LN range from 0.3 to 1.9 g/100 g total fatty acids in human milk samples from the United States, Japan and Germany (4–6). These lipids are found as ~98% triglyceride (TG) and 0.8% PL (6). Commercial infant formulas that are available in the United States contain a ratio of LA to LN and levels of these fatty acids that are similar to breast milk, but most do not contain any preformed AA or DHA. Data indicate that conversion of LA and LN to AA and DHA, respectively, by the desaturation-elongation pathway may not be sufficient to support the needs of growing infants (7–9). Cunnane et al. (7) estimated that formula-fed infants accumulate only half of the DHA that breast-fed infants accrete over the first 6 mo of life; therefore, supplementation with DHA in conjunction with AA is considered necessary to support proper growth and development during this early and rapid growth phase of life. Brain tissue from postmortem infants in the United Kingdom and Australia fed formulas lacking AA

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⁴ Abbreviations used: AA, arachidonic acid; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CNTL, control; DHA, docosahexaenoic acid; EFA, essential fatty acid; EFAD, EFA deficient; FAME, fatty acid methyl ester; HDL, high-density lipoprotein; LA, linoleic acid; LCPUFA, long-chain polyunsaturated fatty acid; LDL, low-density lipoprotein; LN, α -linolenic acid; PL, phospholipid; TG, triglyceride.

and DHA had lower levels of DHA than infants fed breast milk (10–12).

Breast milk is considered the “gold standard” for formula composition. Therefore, inclusion of preformed AA and DHA into infant formula has become a major research focus in infant nutrition. Some studies demonstrate that infants fed formulas supplemented with AA and DHA show improved visual acuity, better neurodevelopment and a lower incidence of necrotizing enterocolitis (13–15). Less research has focused on the bioavailability and metabolism of these long-chain polyunsaturated fatty acids (LCPUFA) in ingredients that would be of practical use in infant formulas.

Presently there are two primary types of preformed AA and DHA that may be used as supplemental sources of LCPUFA in infant formula: a TG source that is produced from single-cell microorganisms (AA from fungi and DHA from microalgae) and a PL source that is extracted from egg yolk oil. The differences in absorption and metabolism of these two sources have received little attention. Recently, Amate et al. (16) conducted a trial comparing the two sources of LCPUFA, PL vs TG, in piglets and reported plasma concentrations of the lipoproteins as well as the composition of the intestinal mucosa. The lipid composition of the jejunal mucosa was not affected by the TG and PL sources, but the sources had different effects on high-density lipoprotein (HDL) vs low-density lipoprotein (LDL). The egg PL diet increased AA and DHA in the HDL PL, whereas the TG diet increased AA and DHA in the LDL PL. The piglet has proven to be a suitable model for comparison to the human infant when studying lipid nutrition. The piglet has many similarities with human infants, including a likeness in the development of the intestine, similar fat digestion and absorption and also many of the pathways of lipid metabolism (17). The purpose of this study was to examine the utilization of algal/fungal TG oils in comparison to an egg PL source and to conduct a stringent assessment of the safety and efficacy of these lipids as delivery sources of LCPUFA.

MATERIALS AND METHODS

Animal care

General. The Institutional Animal Care and Use Committee of North Carolina State University (Raleigh, NC) approved all procedures. A total of 48 piglets from 13 sows were obtained from the North Carolina State University Swine Educational Facility (Raleigh, NC) and were moved to the Grinnell Intensive Swine Research Laboratory (Raleigh, NC) at ~1 d of age. Pigs were placed in individual cages in an environmentally controlled room (32°C) and were trained to consume a liquid diet from a gravity flow feeding system adapted from McClellan et al. (18). The feeding system consisted of bottles suspended above the cages with tubing connecting the bottle to the permanently affixed nipple. All pigs were routinely consuming the liquid diet after 12–16 h of training and were then randomly assigned to one of five dietary treatments. Treatments groups were as follows (Table 1) (19): 1) piglet formula without any preformed AA or DHA added, but adequate amounts of LA and LN (CNTL, $n = 10$); 2) piglet formula plus AA and DHA from egg PL ($n = 10$; Ovothin; Lucas Meyer, Decatur, IL); 3) piglet formula plus AA and DHA from the fungal and algal TG oils ($n = 10$; Martek Biosciences, Columbia, MD); 4) piglet formula plus AA and DHA from the fungal and algal TG oils (Martek Biosciences) with additional choline, cholesterol and soy lecithin PL (American Lecithin, Oxford, CT) to match the PL formula (TG + PL, $n = 10$); 5) piglet formula deficient in EFA (EFAD; <2 g/100 g total fat as LA and devoid of LN; $n = 8$). Formulas with LCPUFA provided 0.6 g/100 g of fatty acids as AA and 0.3 g/100 g as DHA. Fatty acid composition of the diets is presented in Table 2. Another 13 piglets from two litters remained with the sows for the duration of the study. At the

TABLE 1

Composition and calculated analysis of the formula diets fed to piglets, comparing triglyceride (TG) and phospholipid (PL) sources of arachidonic acid (AA) and docosahexaenoic acid (DHA)¹

Ingredient, g/kg	Diet				
	CNTL	TG	TG + PL	PL	EFAD
Mead Johnson oil blend ²	180	182	182	97	0
Martek ARASCO (AA) ³	0	4.4	4.4	0	0
Martek DHASCO (DHA) ³	0	2.1	2.1	0	0
Ovothin 120 (AA & DHA) ⁴	0	0	0	85	0
Soybean oil	110	101.5	69	105	0
Flaxseed oil	0	0	0.8	3	0
Coconut oil (nonhydrogenated)	0	0	0	0	290
Powdered lecithin ⁵	0	0	34	0	0
Choline chloride	0	0	10.6	0	0
Cholesterol	0	0	4.5	0	0
Sodium caseinate ⁶	128	131	131	128	128
Whey protein concentrate ⁷	86	89	89	86	86
Skim milk ⁸	388	373	373	388	388
Lactose	50	59	37	55	50
CaCO ₃	5	5	8.7	8.5	5
Dicalcium phosphate	27	27	27	19	27
Mineral premix ⁹	5	5	5	5	5
Vitamin premix ¹⁰	1.3	1.3	1.3	1.3	1.3
Lysolecithin	10	10	10	10	10
Xanthan gum	10	10	10	10	10
Calculated analysis ¹¹					
ME, kJ/kg ¹²	5053	5053	4977	5067	4783
Fat, g/100 g	30	30	30	30	30
Crude protein, g/100 g	32	32	32	32	32
Lactose, g/100 g	26	26	24	25	26
Calcium:phosphorus	1.4	1.4	1.4	1.4	1.4
PL, g/100 g	0.2	0.2	2.7	2.7	0
TG, g/100 g	29.2	29.2	26.2	26.1	29.4
Cholesterol, g/100 g	0	0	0.43	0.43	0
Phosphatidylcholine, g/100 g	0	0	1.9	1.9	0
Phosphatidylethanolamine, g/100 g	0	0	0.7	0.5	0
Ratio of LA:LN	7.7	7.6	7.7	7.7	—
Calculated g/100 g of total fat					
LA	30.3	29.0	29.9	29.9	1.3
LN	3.9	3.8	3.9	3.9	0
AA	0	0.6	0.6	0.6	0
DHA	0	0.3	0.3	0.3	0

¹ Expressed on an air-dry weight basis.

² Mead Johnson oil blend of palm olein, soy, coconut and high oleic sunflower oils (Mead Johnson Nutritionals).

³ Martek ARASCO and DHASCO (Martek Biosciences).

⁴ Ovothin 120 (Lucas Meyer).

⁵ Powdered soy lecithin (Alcolec F100; American Lecithin).

⁶ Sodium caseinate (International Ingredient, St. Louis, MO).

⁷ Whey protein concentrate (AMP 80; Proliant, Ames, IA).

⁸ Skim milk (Milk Specialties, Dundee, IL).

⁹ Mineral premix (Milk Specialties) contained 1.002 g/100 g Ca, 0.549 g/100 g P, 0.284 g/100 g Na, 0.040 g/100 g Cl, 2.024 g/100 g K, 0.102 g/100 g Mg, 20,000 µg/g Fe, 200 µg/g Co, 1850 µg/g Cu, 400 µg/g I, 5000 µg/g Mn, 60 µg/g Se, 23,500 µg/g Zn.

¹⁰ Vitamin premix (Milk Specialties) contained 33,000,000 IU/kg vitamin A, 6,600,000 IU/kg cholecalciferol, 55,000 IU/kg α-tocopherol, 257,400 µg/g ascorbic acid, 29,983 µg/g D-pantothenic acid, 33,069 µg/g niacin, 8378 µg/g riboflavin, 5115 µg/g menadione, 66 µg/g biotin, 44,000 µg/g vitamin B-12, 2038 µg/g thiamin, 3996 µg/g vitamin B-6, 2756 µg/g folic acid.

¹¹ Calculated analysis based on analysis provided by companies furnishing product and standard feed tables (19).

¹² ME, Metabolizable energy as estimated from book values and information provided by companies supplying ingredients. CNTL, control; TG, triglyceride; PL, phospholipid; EFAD, essential fatty acid deficient; LA, linoleic acid; AA, arachidonic acid; LN, α-linoleic acid; DHA, docosahexaenoic acid.

TABLE 2

Calculated fatty acid composition of diets

Fatty acid	CNTL	TG	TG + PL	PL	EFAD
<i>g/100 g diet</i>					
8:0	0.07	0.07	0.07	0.07	0.07
10:0	0.00	0.00	0.00	0.00	1.57
12:0	0.00	0.01	0.01	0.00	13.78
14:0	0.00	0.04	0.04	0.00	5.63
14:1	0.04	0.04	0.04	0.04	0.04
16:0	5.08	5.12	5.48	5.38	2.93
17:1	0.18	0.18	0.18	0.18	0.18
18:0	0.50	0.51	0.54	1.46	0.73
18:1	9.34	9.31	8.92	9.30	2.23
18:2(<i>n</i> -6)	9.09	8.69	8.94	8.98	0.38
18:3(<i>n</i> -3)	1.18	1.13	1.17	1.16	0.00
20:3	0.00	0.02	0.02	0.00	0.00
20:4(<i>n</i> -6)	0.00	0.18	0.18	0.18	0.00
22:0	0.00	0.01	0.01	0.00	0.00
22:6(<i>n</i> -3)	0.00	0.09	0.09	0.09	0.00
24:0	0.00	0.01	0.01	0.00	0.00

end of the study, piglets were killed with an American Veterinary Medical Association-approved electrocution device followed by exsanguination (laceration of the brachiocephalic arteries) and tissues were collected. An initial group of 10 piglets from five litters was also used to detect any changes that occurred because of treatments.

Animal feeding and diets. Diets were reconstituted at 150 g per liter of water (~11 g/100 g dry matter). Formula was added four times daily (0800, 1300, 1800 and 2300 h) to ensure freshness and to provide pigs free access. All components of the feeding system were cleaned thoroughly each day before the first feeding (0800 h) with a liquid chlorinated detergent (DS Liquid, Command; Diversey Corp., Wyandotte, MI). Formula was reconstituted on a daily basis and stored at 4°C until fed.

Cobalt EDTA was prepared as described by Uden et al. (20) and was added to diets (0.1 g/100 g of dry diet) ~36 h before removal of pigs from the experiment as an inert marker of dry matter digestibility.

Rationale. The objective of this study was to determine the efficacy and safety of the algal/fungal TG source of supplemental AA and DHA in neonatal piglets. The TG source also was compared with the currently used egg PL source of AA and DHA. The TG + PL group was included to determine whether the potential deleterious effects of elevated PL on intestinal health were due to PL in general or were specific to the PL source of AA and DHA. The initial piglets served as a beginning reference point for comparison with all treatments. The sow-reared piglets, which remained on the sow for the entire study and received the ideal source of nutrients for the pig, were used as a comparison for all formula-fed groups. The EFAD diet was included to serve as a negative control to illustrate the responsiveness of the model to poor EFA status.

Sample collection and analytical procedures

Performance and blood collection. Formula intake was determined gravimetrically on a daily basis. Pigs were weighed daily and blood was collected via jugular venipuncture on d 0, 8 and 16 of the study at 0900 h after all piglets had been fed. After collection, blood samples were centrifuged (model 64000; Sorvall, Newtown, CT) at 825 × g for 10 min at 4°C. Plasma was collected and aliquots were frozen at -80°C until fatty acid analysis. On d 16, an additional blood sample was taken and 17 blood metabolites were measured by a VetScreen (Antech Diagnostics, Farmingdale, NY). These variables were measured to investigate the clinical safety of the LCPUFA sources. The VetScreen 17 measured glucose, blood urea nitrogen (BUN), creatinine, total protein, albumin, total bilirubin, alkaline phosphatase, alanine aminotransferase (ALT), aspartate aminotransferase (AST), cholesterol, calcium, phosphorus, sodium, potassium,

chloride, the albumin:globulin ratio, the BUN:creatinine ratio and globulin.

Fatty acid analysis. Plasma lipids were extracted using the method of Bligh and Dyer (21) and fatty acid methyl esters (FAME) were produced using the method of Morrison and Smith (22). FAME were analyzed by gas-liquid chromatography using an Agilent 5890-Plus (Hewlett-Packard, Palo Alto, CA) equipped with a flame ionization detector. The FAME were separated on a 30-m FAMEWAX capillary column (0.25-mm diameter, 0.25- μ m coating thickness; Restek, Bellefonte, PA) using helium at a flow rate of 2.1 mL/min with a split ratio of 48:1. The chromatographic analysis parameters included an oven starting temperature of 130°C that was increased at 6°C/min to 225°C, where it was held for 20 min before increasing to 250°C at 15°C/min, with a final hold of 5 min. The injector and detector temperatures were constant at 220 and 230°C, respectively. Peaks were identified by comparison of retention times with external FAME standard mixtures from NuCheck Prep (Elysian, MN). The fatty acid profiles were expressed as grams per 100 g of total fatty acids.

Tissue collection and analytical procedures. Immediately after exsanguination, the abdomen was opened and the gastrointestinal tract was removed from the gastroesophageal junction to the distal end of the rectum. The jejunum and ileum were separated from the duodenum, stomach and mesentery from the peritoneal inflection to the ileocecal junction. The anterior and posterior ends of the removed small intestinal segment were noted and the jejunum and ileum were laid in 60-cm serpentine loops. The midpoint was marked, and intestine proximal and distal to this midpoint was considered jejunum and ileum, respectively. At approximately mid-jejunum and mid-ileum, two adjacent segments, one 3-cm and another one slightly larger than 10-cm in length, were removed. Digesta contents were taken from the distal ileum and frozen (-20°C) for dry matter digestibility analysis (23). Lactase specific activity was measured using the method of Dahlqvist (24) as modified by Oliver et al. (25). The 3-cm intestinal segments were processed, embedded and stained according to procedures described by Luna (26) as reported in Oliver et al. (25) for measurement of villi height and width as well as crypt depth. Invasive measures were included because a reduction in villi height and lactase specific activity and an increase in crypt depths would indicate deleterious effects on the intestine.

Both the liver and the spleen were removed from the abdomen and weighed. Two pieces of liver were collected from the same two liver lobes in each piglet. Samples were fixed in 10% neutral buffered formalin for at least 48 h. Fixed liver was routinely processed, embedded in paraffin, sectioned at 6- μ m thickness and stained with hematoxylin and eosin for histologic review.

Liver sections were evaluated and graded using a subjective scale by a board-certified veterinary pathologist at North Carolina State University College of Veterinary Medicine (Raleigh, NC), who was unaware of the treatment groups. Cytoplasmic vacuoles were interpreted as either glycogen containing or lipid containing based on their histologic appearance and then scored on a four-point scale of scant, mild, moderate or extensive. Other features noted were inflammation, extramedullary hematopoiesis and hemosiderin deposition. Again, these invasive procedures allowed for the further investigation of the safety of the supplemental sources for AA and DHA.

Statistical analysis

Values in the text are means \pm SEM. SAS (SAS Institute, Cary, NC) Proc GLM procedure was used for statistical analysis appropriate for a completely randomized design. Treatment differences were evaluated using a protected least significant differences, which provided all pairwise comparisons. Differences were deemed significant when $P < 0.05$.

Liver histology data were analyzed using StatXact software (version 3.1; Cytel Software, Cambridge, MA). The Kruskal-Wallis test was used to examine vacuolization liver data and differences were deemed significant when $P < 0.05$.

RESULTS

Performance, growth and food intake

Over the treatment period, there were no differences in piglet body weights (data not shown) except on d 16, when the TG piglets were heavier than the EFAD piglets (7599 ± 304 g vs 6562 ± 340 g; $P < 0.05$). Formula-fed piglets gained 324 ± 17 g/d throughout the study period, with the TG piglets having a greater gain than the EFAD piglets (346 ± 17 vs 293 ± 18 g/d; $P < 0.05$). The sow-reared piglets gained 297 ± 29 g/d. Daily feed intake and feed efficiency (grams of gain per gram of feed) did not differ among groups for the 16-d period and were 1953 ± 68 g/d and 1.58 ± 0.06 , respectively.

Small intestinal morphology

Jejunal and ileal villi height decreased from d 0 to d 16 in all treatments as assessed by comparison with the initial piglets (Fig. 1; $P < 0.0001$). There were no differences in jejunal villi height among any of the formula-fed groups, nor were they different from the sow-reared piglets on d 16. CNTL, TG, TG + PL, and EFAD piglets all had similar ileal villi height, whereas PL piglets had lower ileal villi heights than the CNTL piglets ($P < 0.03$). TG + PL, PL and EFAD piglets had lower ileal villi heights compared with the sow-reared piglets ($P < 0.05$) but did not differ from CNTL or TG piglets. Jejunal and ileal crypt depths were greater in all formula-fed groups at d 16 compared with the initial piglets ($P < 0.03$). Jejunal villi width was greater in the TG, TG + PL, and PL piglets compared with the sow-reared piglets ($P < 0.05$) and both TG + PL and PL piglets had greater widths than the initial piglets ($P < 0.02$, data not presented). Ileal villi width was greater in CNTL, TG, TG + PL, and PL piglets compared with the initial piglets ($P < 0.03$), and both TG + PL and PL piglets had greater villi width than the sow-reared piglets ($P < 0.03$, data not presented).

Enzyme specific activity

The addition of either TG or PL sources of AA and DHA did not affect jejunal or ileal lactase specific activity in any of

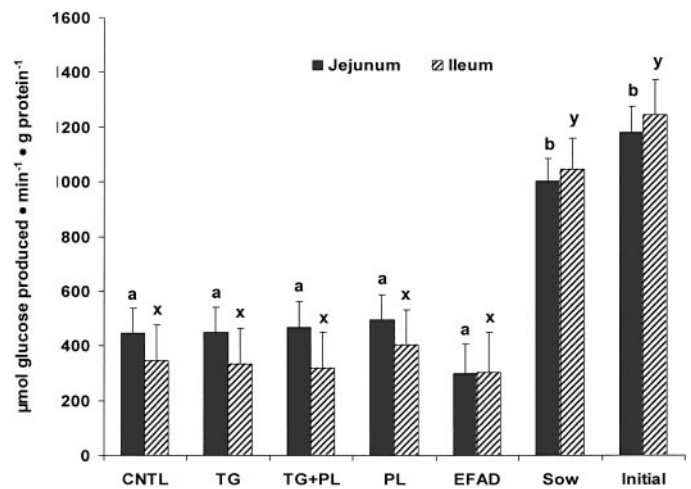


FIGURE 2 Lactase specific activity in the jejunum and ileum of neonatal pigs fed either triglyceride (TG) or phospholipid (PL) sources of arachidonic acid (AA) and docosahexaenoic acid (DHA) for 16 d. Values are means \pm SEM; $n = 8-13$. Initial piglets were killed before treatment initiation at 1 d of age. Bars lacking common letters differ, $P < 0.05$.

the formula-fed piglets (Fig. 2). Both initial and sow-reared piglets had greater lactase specific activity than the formula-fed groups ($P < 0.0001$), but they did not differ from one another.

Dry matter digestibility and digesta dry matter content

Dry matter content of the digesta from the distal rectum was 22.5 ± 1.3 g/100 g and did not differ among dietary treatments (data not shown). Ileal and rectal apparent dry matter digestibilities of the diets were 81.3 ± 2.5 and $89.7 \pm 2\%$, respectively (Fig. 3). Ileal apparent dry matter digestibility did not differ among the CNTL, TG, TG + PL, and EFAD piglets, but the PL piglets had a lower ileal apparent dry matter digestibility than the CNTL and TG piglets ($P < 0.01$).

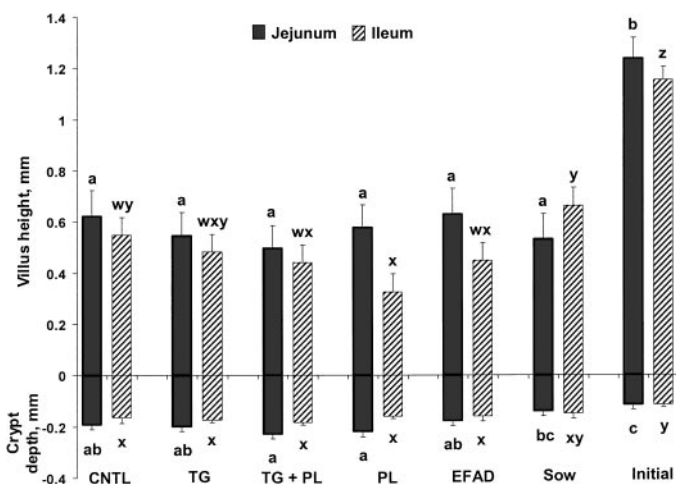


FIGURE 1 Villus heights and crypt depths of pigs fed either triglyceride (TG) or phospholipid (PL) sources of arachidonic acid (AA) and docosahexaenoic acid (DHA) for 16 d. Values are means \pm SEM; $n = 8-13$. Initial piglets were killed before treatment initiation at 1 d of age. Bars lacking common letters differ, $P < 0.05$.

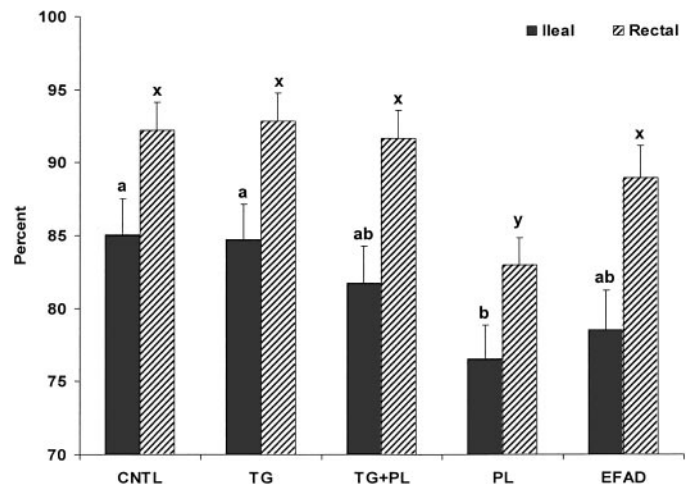


FIGURE 3 Ileal and rectal apparent dry matter digestibilities of neonatal pigs fed either triglyceride (TG) or phospholipid (PL) sources of arachidonic acid (AA) and docosahexaenoic acid (DHA) for 16 d. Values are means \pm SEM; $n = 8-10$. Bars lacking common letters differ, $P < 0.05$.

Liver biochemistry

Plasma ALT and AST were used as indicators of potential liver damage. All formula-fed piglets and the sow-reared piglets had similar ALT activities that were lower than those of the initial piglets (Fig. 4; $P < 0.01$). AST was higher in the initial piglets compared with the CNTL, TG, TG + PL, and EFAD piglets ($P < 0.003$), but in initial piglets this activity did not differ from PL piglets. PL piglets had higher AST activity than the TG + PL piglets and the sow-reared piglets ($P < 0.02$). Relative liver weights were not different in CNTL, TG, TG + PL, and PL piglets ($3.2 \pm 0.1\text{g}/100\text{g}$ body; data not shown). EFAD piglets had greater relative liver weights than all other groups ($3.5 \pm 0.1\text{g}/100\text{g}$ body; $P < 0.03$), whereas the sow-reared piglets had the lower relative weights than all other treatments ($2.5 \pm 0.1\text{g}/100\text{g}$ body; $P < 0.002$). Gross liver histology (Table 3) showed that sow-reared piglets had moderate lipid- and glycogen-containing vacuoles compared with essentially none found in the formula-fed piglets ($P < 0.01$). All treatment groups had signs of both extramedullary hematopoiesis and hemosiderin, but they did not differ from one another ($P = 0.6$). Crude protein percentage of the liver (Table 4) did not differ among groups ($P > 0.06$). Liver lipid did not differ among the CNTL piglets and the LCPUFA-supplemented groups. The percentage of liver lipid was higher in the EFAD and initial piglets than in the CNTL, TG and PL piglets ($P < 0.005$). Piglets in the TG + PL group had proportions of liver lipid not different from the CNTL, TG and PL, sow-reared and initial piglets, but had less liver lipid than the EFAD piglets ($P < 0.005$).

Plasma fatty acids (Table 5)

Piglets that were fed the LCPUFA TG source alone (TG piglets) had higher plasma levels of both AA and DHA than the CNTL piglets (Fig. 5; $P < 0.05$). All three groups that were fed the preformed AA (TG, TG + PL, and PL) had similar plasma AA concentrations that were much higher than those of the EFAD piglets ($P < 0.0001$) but not different from the sow-reared piglets. The TG, TG + PL and sow-reared

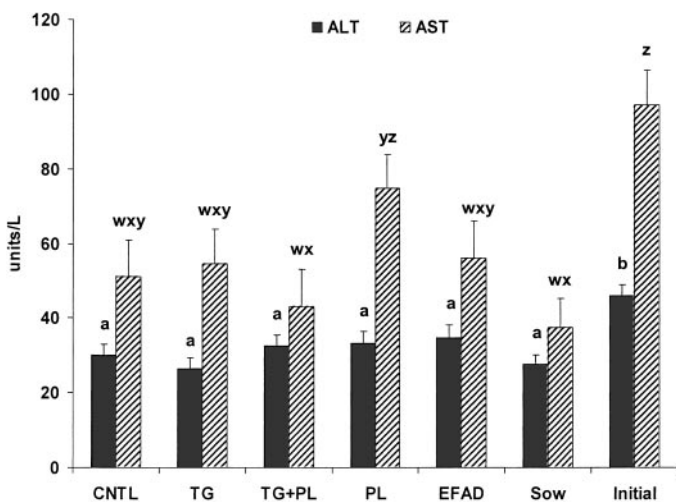


FIGURE 4 Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) values in neonatal pigs fed either triglyceride (TG) or phospholipid (PL) sources of arachidonic acid (AA) and docosahexaenoic acid (DHA) for 16 d. Values are means \pm SEM; $n = 8-13$. Initial piglets were killed before treatment initiation at 1 d of age. Bars lacking common letters differ, $P < 0.05$.

TABLE 3

Gross liver histology in neonatal piglets fed supplemental LCPUFA of arachidonic acid (AA) and docosahexaenoic acid (DHA) in the form of either triglyceride (TG) or phospholipid (PL)¹

Diet group	<i>n</i>	None	Scant	Mild	Moderate	Marked
Lipid in vacuoles²						
CNTL ^a	10	9	1	0	0	0
TG ^a	10	10	0	0	0	0
TG + PL ^a	10	10	0	0	0	0
PL ^a	10	10	0	0	0	0
EFAD ^a	8	8	0	0	0	0
Sow ^b	13	5	1	0	5	2
Initial ^{ab}	10	6	1	3	0	0
Glycogen in vacuoles³						
CNTL ^a	10	0	2	7	1	0
TG ^{ab}	10	0	5	3	2	0
TG + PL ^{ab}	10	0	3	6	1	0
PL ^{ab}	10	0	7	2	1	0
EFAD ^b	8	1	5	2	0	0
Sow ^c	13	0	1	4	6	2
Initial ^{ab}	10	0	3	6	1	0

¹ Values are number of occurrences. Within the first column, treatments lacking common superscripts differ ($P < 0.05$).

² Vacuoles containing lipid in the liver.

³ Vacuoles containing glycogen in the liver.

piglets did not differ from the initial piglets in plasma AA levels. Plasma DHA levels were higher in the TG and TG + PL piglets than in the sow-reared piglets ($P < 0.03$) but did not differ among piglets given LCPUFA-supplemented formulas and initial piglets. Although the groups differed in several plasma fatty acid levels (Table 5), especially the EFAD group, the focus of this study concerned efficacy of different sources of LCPUFA.

Blood biochemistry

BUN concentrations did not differ among the formula-fed groups. Sow-reared piglets had lower BUN ($5.5 \pm 1\text{mmol/L}$) and initial piglets had much higher BUN ($23 \pm 1\text{mmol/L}$) than the formula-fed piglets ($12.3 \pm 1\text{mmol/L}$; $P < 0.0002$).

TABLE 4

Liver crude protein and fat composition of neonatal piglets fed supplemental LCPUFA of arachidonic acid (AA) and docosahexaenoic acid (DHA) in the form of either triglyceride (TG) or phospholipid (PL)¹

Treatment	<i>n</i>	Crude protein ²	Fat
<i>g/100 g</i>			
CNTL	10	71.5 \pm 1.5	12.6 \pm 0.8 ^a
TG	10	73.5 \pm 1.7	12.0 \pm 0.8 ^a
TG + PL	10	70.2 \pm 1.5	13.3 \pm 0.8 ^{ac}
PL	10	71.7 \pm 1.5	12.0 \pm 0.8 ^a
EFAD	8	76.1 \pm 1.9	16.7 \pm 0.9 ^b
Sow	13	68.2 \pm 1.7	13.3 \pm 0.8 ^{ac}
Initial	10	69.8 \pm 1.5	15.2 \pm 0.8 ^{bc}

¹ Tabulated values are means \pm SEM. Within a column, means without a common letter differ, $P < 0.05$.

² Nitrogen \times 6.25.

TABLE 5

Plasma fatty acids in neonatal piglets fed supplemental LCPUFA of arachidonic acid (AA) and docosahexaenoic acid (DHA) in the form of either triacylglycerol or phospholipid (PL)¹

Fatty acid	CNTL	TG	TG + PL	PL	EFAD	Sow	Initial	Pooled SEM
<i>g/100 g total fatty acids</i>								
14:0	0.26bc	0.28b	0.21bc	0.16bc	1.53a	0.31bc	0.08c	0.08
16:0	22.34ab	23.23ab	23.61ab	23.59ab	22.02a	24.11b	15.63c	0.8
16:1	0.14d	0.14d	0.11d	0.22d	1.16a	1.04b	0.46c	0.04
16:3	0.37a	0.43ab	0.31a	0.38a	0.53b	0.36a	0.35a	0.04
18:0	20.87acd	19.87cd	21.86abc	22.39ab	23.36b	19.50d	23.62b	0.9
18:1(n-9)	13.47a	13.40a	12.31ac	11.91ac	20.18b	11.63c	19.58b	0.6
18:1(n-7)	1.48a	1.47a	1.70ab	1.89bc	1.65a	1.97c	2.99d	0.09
18:2	28.24a	24.58c	24.09c	25.22c	19.78b	24.90c	17.12d	0.8
18:3(n-6)	0.09a	0.07a	0.08a	0.10a	0.20b	0.04a	0.18b	0.03
18:3(n-3)	0.72a	0.68a	0.54bd	0.65ad	0.47b	0.32c	0.56d	0.04
20:0	0.08a	0.10a	0.12a	0.10a	0.10a	0b	0b	0.03
20:1(n-9)	0.05a	0.10ab	0.05a	0.07a	0.08a	0.06a	0.14b	0.02
20:2	0.34a	0.36a	0.30a	0.37a	2.34b	0.31a	0.31a	0.06
20:3	0.30a	0.35a	0.32a	0.28a	0.72b	0.58c	1.48d	0.04
20:4(n-6)	7.49a	9.85b	9.72ab	8.56ab	3.44c	9.56ab	11.91d	0.8
20:5	0.21ad	0.24ab	0.24ab	0.30b	0.51c	0.14d	0.28a	0.03
22:0	0.09ad	0.12ab	0.15b	0.03c	0.08ad	0.07ac	0.05cd	0.02
22:4	0.25a	0.31a	0.25a	0.25a	0.19a	0.85b	0.58c	0.05
22:5	1.00ac	0.77a	0.85a	0.84a	0.32b	1.20c	0.72a	0.1
22:6(n-3)	1.76ad	3.02b	2.72ab	2.13abd	0.58c	1.67d	2.37abd	0.3
24:0	0.07a	0.08ab	0.06a	0.04a	0.06a	0.18b	0.05a	0.03
24:1	0a	0.06abd	0.03abd	0.09bd	0.02abd	0.19c	0.08d	0.03
Other ²	0.06	0.11	0.07	0.06	0.27	0.63	0.94	0.02

¹ Within a row, means without a common letter differ, $P < 0.05$.

² Trace amounts of 14:1, 15:0, 15:1, 20:3n3, 22:1, 22:2 and 22:3 were also detected in some of the samples. These fatty acids were not statistically analyzed because of the trivial amounts represented.

Plasma cholesterol levels were higher in the sow-reared piglets than in all other groups ($P < 0.03$), whereas the initial piglets had the lowest plasma cholesterol levels ($P < 0.03$). The

CNTL, TG, TG + PL, and PL piglets did not differ in plasma cholesterol (data not shown). Plasma glucose did not differ among the CNTL, EFAD, TG and TG + PL piglets ($P > 0.08$) but was lower in the PL piglets than in the CNTL, TG and TG + PL piglets ($P < 0.04$, data not shown).

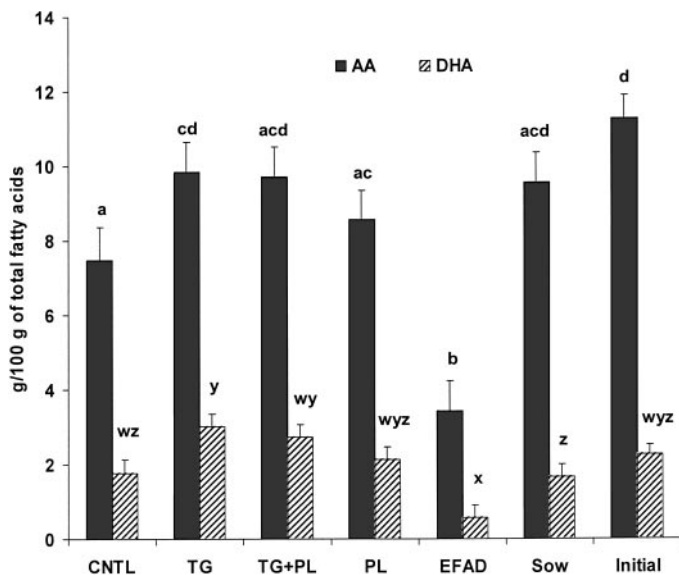


FIGURE 5 Arachidonic acid (AA) and docosahexaenoic acid (DHA) concentrations expressed as g/100 g total plasma lipid fatty acids in neonatal pigs fed either triglyceride (TG) or phospholipid (PL) sources of AA and DHA for 16 d. Values are means \pm SEM; $n = 8-13$. Initial piglets were killed before treatment initiation at 1 d of age. Bars lacking common letters differ, $P < 0.05$.

DISCUSSION

Over the last decade, much research has focused on the need for inclusion of LCPUFA, AA and DHA in infant formulas in the United States using both animal models such as the piglet (13,15,16,27-29) as well as human clinical trials (13,15,16,27,28). The piglet model has proven to be an appropriate and useful tool when making comparisons to human infants. The interest in supplementation stems from these LCPUFA being important to perinatal retinal and central nervous system growth and development. As stated previously, the composition of human milk has a TG content of ~98% of total lipid, whereas only ~0.8% is PL (6). To date there have been few comparisons of the two sources, TG and PL, especially with regard to the gastrointestinal tract and how the sources are digested and absorbed.

In this study, our primary objective was to investigate the use of novel algal and fungal TG sources for the supplementation of the LCPUFA, AA and DHA in infant formulas, and to perform a rigorous evaluation of the safety and efficacy of these substances as well as a comparison with the PL source. After 16 d of supplementation we determined that neither source of LCPUFA affected piglet growth rate, formula intake, plasma cholesterol or BUN. Our data are similar to previously reported studies in that growth rates and intakes were consistent for both the CNTL and the LCPUFA-supplemented

groups (16,28), and plasma cholesterol and BUN levels were similar to the results published by Huang et al. (30).

To date, no other study evaluating either the TG or PL source of LCPUFA together or separately has investigated their effects on small intestine health. Small intestinal morphology has typically been used as an estimate of intestinal health in pigs (31–34). Lopez-Pedrosa et al. (35) used dietary PL to speed repair of the small intestine in malnourished piglets. However, it is difficult to compare these results to ours because the piglets were undernourished and had different jejunal and ileal morphology than the piglets in our study. Also, the levels of AA and DHA were lower than those used in the present study, and the PL load was 2.5% of the formula, which is slightly less than the 2.7% of the formula that was used in the present study. Amate et al. (16) measured the lipid composition of the jejunum mucosa and found no differences between the TG and PL sources of LCPUFA. In the current study we found no differences in jejunal villi height across any of the formula-fed pigs compared with the sow-reared piglets. However, ileal villi height in the PL-supplemented piglets was 40% shorter than in the CNTL piglets, whereas the TG and TG + PL piglets did not differ from the CNTL piglets. This villi shortening is associated with a decreased absorptive area for the PL piglets. The sow-reared piglets had greater ileal villi height than the PL, TG + PL, and EFAD piglets. Jejunal crypt depths showed similar results, as the sow-reared piglets had less crypt depth than the PL or TG + PL piglets; however, these changes were small and probably not physiologically important. Because of the limited research that has been conducted on intestinal morphology when comparing the TG and PL sources of LCPUFA, there are no data for comparison with these results. However, values reported for the CNTL, TG and sow-reared piglets are similar to other research conducted in our laboratory evaluating intestinal morphology in piglets that ate ad libitum (25).

Intestinal lactase activity is high at birth and reaches maximum activity at ~1 wk of age in piglets (36–38). In the current study the supplementation of LCPUFA in the form of TG or PL did not affect lactase specific activity. Comparatively, the sow-reared piglets had one- to twofold higher lactase specific activity compared with all other formula-fed treatments. The inability of diet to affect lactase activity is well documented (39–42). However, the decrease in lactase specific activity seen in the formula-fed piglets compared with the sow-reared piglets could be due to environmental factors and/or differences between sow's milk and milk ingredients derived from bovine sources.

The apparent ileal and rectal dry matter digestibilities of the diets was greater in the TG piglets ($84.7 \pm 2\%$) compared with the PL piglets ($76.5 \pm 2\%$). No other studies have evaluated the dry matter digestibility of diets containing LCPUFA, but we found that the addition of the PL source of AA and DHA decreased the rectal apparent dry matter digestibility compared with all other treatments ($P < 0.04$). The TG + PL piglets had similar ileal dry matter digestibility to the PL piglets, but rectal dry matter digestibility of the TG + PL piglets was similar to the CNTL and TG piglets in that it was higher than in the PL piglets. Amate et al. (43) measured fat apparent absorption in rats after feeding two PL LCPUFA sources. Compared with a fish oil TG source the pig brain PL source was absorbed less, but when both LCPUFA sources were from egg the PL did not differ from the TG source. Thus, they concluded that the absorption of a PL or TG source depends on the characteristics of the individual fat source. Carnielli et al. (44) measured absorption of TG and PL LCPUFA in supplemented formulas as well as in preterm

breast milk in preterm infants. They found better absorption of the DHA from the PL source compared with either the TG source or preterm breast milk but no difference in AA absorption among the three groups. The discrepancy between the difference in digestibility and/or absorption of the two different sources of LCPUFA may be due in part to a difference in the gastrointestinal tract maturity, which is much less in the premature infant compared with the term piglet (45). However, gastrointestinal tract maturity of preterm and term piglets was not examined in the current study.

Total plasma lipid AA and DHA concentrations were reflective of the inclusion of LCPUFA in the diets of the TG, TG + PL, and PL piglets. The rise in plasma AA and DHA after supplementation is similar to what has been seen previously in preterm infants fed formulas with increasing levels of added AA and DHA from the algal/fungal TG sources (46). In our study, there were no differences in plasma DHA or AA detected among any of the LCPUFA-supplemented groups. The TG piglets had a greater percentage of plasma AA and DHA compared with both the CNTL and the EFAD piglets. However, the TG + PL piglets and the PL piglets did not differ from the CNTL piglets, suggesting that the dietary PL load in the PL and TG + PL formulas decreased the absorption of the LCPUFA. Amate et al. (16) found that in piglets the TG source of LCPUFA increased the AA and DHA in LDL PL but that the PL source increased the AA and DHA in HDL PL, suggesting that the two sources might follow different transport pathways. Other studies in human infants that examined either TG (13) or PL (15,47) sources found an increase in plasma and/or erythrocyte AA and DHA compared with a nonsupplemented group, but few studies have compared the two sources to one another (16,44).

The liver is a metabolically sensitive organ that may be examined to determine potential side effects of the metabolism of different sources of LCPUFA. Limited research has focused on the liver, with the exception of Huang et al. (30), who measured liver weights, both absolute and relative to individual body weight, and also liver histology in piglets fed increasing levels of the algal and fungal TG sources of LCPUFA. As seen in the Huang et al. (30) study, LCPUFA-supplemented piglets had similar liver weights compared with the CNTL piglets. The sow-reared piglets had the lowest liver weights, which may be due to their lower intakes compared with the formula-fed groups. Liver histology did not differ between the LCPUFA-supplemented piglets or when compared with the CNTL piglets, again similar to the results of Huang et al. (30). The sow-reared piglets had a greater frequency of both lipid- and glycogen-containing vacuoles in the liver compared with all other treatments, which could be indicative of the high level of fat in sow's milk. The proportion of liver crude protein did not differ among treatments, but lipid content varied. The CNTL, TG and PL piglets had the lowest lipid percentage, whereas the initial piglets and the EFAD piglets had the highest lipid percentage. All piglets were fed similar levels of fat, but the metabolism of short and medium chain saturated fatty acids is quite different from that of LCPUFA and thus might explain the increase in liver lipid in the EFAD piglets. Other effects of EFA deficiency, such as a change in lipid transport out of the liver, also may have altered liver fat content. Two other markers of putative cellular damage are the enzymes ALT and AST. There were no differences in ALT; however, AST was greater in PL piglets than in TG + PL piglets. There are several factors that could explain this increase in AST, such as intestinal injury; PL pigs experienced villi shortening during the study period. The AST levels in the PL piglets did not differ from that found in the CNTL or the

TG piglets, but AST was higher than in the sow-reared piglets. Huang et al. (30) also measured these enzymes and reported no difference between treatment groups, but the data represent only the algal/fungal TG sources of the LCPUFA. Therefore, further study of the liver enzymes is needed to determine the reason for the elevated AST in the PL group.

In conclusion, the results of this study show that the TG form of the LCPUFA, AA and DHA may be more efficacious than the PL source based on increases in both plasma AA and DHA concentrations compared with CNTL piglets. The TG sources may also be more appropriate based on effects of the PL source on small intestinal morphology, apparent dry matter digestibility and elevated AST levels. The purpose of this study was to investigate these novel sources of supplemental AA and DHA, and from the variables measured the TG sources appear to be safe and efficacious for use in human neonates as demonstrated using the piglet model. Supplementation of infant formula with the TG source of AA and DHA also helps to maintain the overall balance of TG and PL in formula that is typically found in human milk.

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