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Safety assessment of EPA + DHA canola oil by fatty acid profile comparison to various edible oils and fat-containing foods and a 28-day repeated dose toxicity study in rats

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ABSTRACT

The omega-3 long-chain polyunsaturated fatty acids (LC-PUFAs) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are recognized for their health-promoting qualities. Marine fish and fish oil currently provide the main sources of EPA and DHA for human consumption. An alternative plant-based source of EPA and DHA is provided by EPA + DHA canola event LBFLFK (LBFLFK). A comparative analysis and a 28-day toxicity study assessed the safety of LBFLFK refined, bleached, and deodorized (RBD) oil. Thirty-one different commercially-obtained fat and oil samples were tested, and principal component analysis showed that the overall fatty acid profile of LBFLFK RBD oil was most similar to *Mortierella alpina* oil and salmon flesh. Samples with the fewest differences in the presence or absence of individual fatty acids compared to LBFLFK RBD oil were menhaden oil and some other fish oils. In a 28-day toxicity study, LBFLFK RBD oil was administered by oral gavage to male and female Wistar rats. No signs of toxicity were evident and no adverse effects were noted in clinical observations, clinical pathology, or histopathology. Overall, these studies support the safety of LBFLFK RBD oil as a source of EPA and DHA for human consumption.

1. Introduction

The health benefits of consuming omega-3 long-chain polyunsaturated fatty acids (LC-PUFAs), such as eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3), are well established (Calder, 2014, 2017; Kitessa et al., 2014; Sprague et al., 2016). The United States (U.S.) Food and Drug Administration (FDA) (U.S. FDA, 2003), Health Canada's Food Directorate (Health Canada, 2016), and European Food Safety Authority (EFSA) (EFSA Panel on Dietetic Products et al., 2009, 2010, 2011, 2012) allow qualified health claims to be made on products containing EPA and DHA. Furthermore, numerous organizations, including the American Heart Association, the Food and Agriculture Organization/World Health Organization, the Heart Foundation, Health Canada, and the British Nutrition Foundation, generally recommend intakes of 250–500 mg of combined EPA and DHA per day. While this recommended daily dose is met in some countries, many countries, including the U.S., fall below the recommended average daily intake (Gebauer et al., 2006; Kris-Etherton et al., 2009; Flock et al., 2013; Salem and Eggersdorfer, 2015).

Current human consumption of EPA and DHA is primarily through seafood, including finfish (e.g., salmon, tuna, and cod) and shellfish (e.g., crab, mussels, and oysters), with a large portion provided by aquaculture. Like humans, most fish synthesize very little EPA and DHA and instead accumulate these fatty acids through their diets. Therefore, about 75–85% of the global production of fish oil is actually used by the aquaculture industry in feed preparation (Tacon and Metian, 2008; Tocher, 2015). To meet a recommended daily intake of 500 mg of combined EPA and DHA per person per day for the global population using current sources, the annual global human consumption of fish oil would need to be approximately 1.3 million metric tons. However, producing such a volume of these fatty acids presents a significant challenge given that the current global EPA and DHA production from all sources is only about 0.5 million metric tons (Salem and Eggersdorfer, 2015). Furthermore, global production of fish oil is not

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Abbreviations

ALB	albumin
ALP	alkaline phosphatase
ALT	alanine aminotransferase
AST	aspartate aminotransferase
BASOA	basophils absolute
CA	calcium
CHOL	cholesterol
CL	chloride
CREA	creatinine
DHA	docosahexaenoic acid
EFSA	European Food Safety Authority
EOSA	eosinophils absolute
EPA	eicosapentaenoic acid
FDA	Food and Drug Administration
GGT	gamma-glutamyl transferase
GLOB	globulins
GLP	Good Laboratory Practice
GLUC	glucose
GRAS	Generally Recognized as Safe
HCT	hematocrit
HGB	hemoglobin
HQT	prothrombin time
INP	inorganic phosphate
K	potassium
LBFLFK	EPA + DHA canola event LBFLFK
LC-PUFA	long-chain polyunsaturated fatty acid
LOQ	limit of quantitation
LYMPHA	lymphocytes absolute
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MCV	mean corpuscular volume
MONOA	monocytes absolute
NA	sodium
NEUTA	polymorphonuclear neutrophils absolute
OECD	Organization for Economic Co-operation and Development
PC	principal component
PLT	blood platelet count
RBC	red blood cells
RBD	refined, bleached, and deodorized
RETA	reticulocytes
SD	standard deviation
TBA	total bile acids
TBIL	total bilirubin
TPROT	total protein
TRIG	triglycerides
U.S	United States
UREA	urea
WBC	white blood cells

expected to increase beyond current levels due to sustainability limits (Tocher, 2015). Therefore, the need for additional sources of EPA and DHA has become the primary focus of many public and private research organizations (Kitessa et al., 2014; Tocher, 2015).

EPA and DHA-rich food products and dietary supplements have been extensively reviewed for safety by various agencies and are currently available for consumers. Many products containing EPA and DHA are considered Generally Recognized as Safe (GRAS) after review by the U.S. FDA, and more specifically, EPA and DHA from menhaden oil are qualified as GRAS up to 3 g per person per day (U.S. FDA, 2017). The primary sources of EPA and DHA supplements are refined fish oils. Other sources like krill oil, fermented microalgal oils, and biotechnology-derived yeast oils are also used, but in 2013 these oils only accounted for 3% of the total volume consumed (Kris-Etherton et al., 2009; Blasbalg et al., 2011; Rice and Ismail, 2016). Recent efforts have focused on engineered oil seed crops as plant-based, sustainable, and scalable production systems for increasing the availability of EPA and DHA for aquaculture and human food or dietary supplements (Petrie et al., 2014; Senger et al., 2016; Walsh et al., 2016; Sprague et al., 2017; Usher et al., 2017).

The EPA + DHA canola event LBFLFK (LBFLFK) is a biotechnology-derived canola (*Brassica napus*) plant line developed by BASF Plant Science, L.P. that contains genes that impact the content of omega-3 LC-PUFAs in the seeds and also contains a gene that confers tolerance to imidazolinone herbicides. In the seeds of LBFLFK, LC-PUFA biosynthesis is conferred by the introduction of a metabolic pathway consisting of ten genes that encode seven desaturases and three elongases to produce EPA and DHA from endogenous canola oleic acid (C18:1n-9). A previous study characterized the substrate specificities of each of the ten enzymes that were introduced into LBFLFK (Yilmaz et al., 2017). Substrates tested included endogenous canola fatty acids and the products of each introduced enzyme. The results from this comprehensive enzyme substrate-specificity study showed the complete repertoire of fatty acid products that can be generated by the network of introduced elongases and desaturases. Fig. 1 depicts the biosynthetic pathway in LBFLFK that has been introduced.

Herein, we present a safety assessment of the fatty acids contained in LBFLFK refined, bleached, and deodorized (RBD) oil, which is food-grade quality oil produced from LBFLFK grain. Included in the assessment are both the endogenous fatty acids in LBFLFK with altered levels outside of the range of conventional canola varieties and those fatty acids not present in conventional canola varieties but synthesized in LBFLFK. For this evaluation, two complementary approaches were performed. First, the fatty acid profile of LBFLFK RBD oil was compared to various edible oils and fat-containing foods that are safely and routinely consumed by humans. Second, the safety of LBFLFK RBD oil in a 28-day repeated dose toxicity study in Wistar rats was assessed.

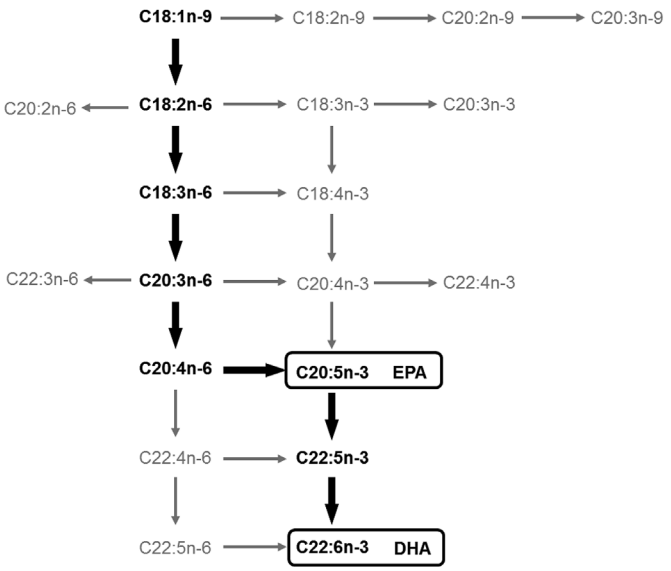

Fig. 1. EPA and DHA biosynthetic pathway in LBFLFK. Each arrow represents an enzymatic activity introduced into canola. The primary route of synthesis, based on enzyme substrate preferences, is indicated by bold black arrows. Gray arrows represent additional reactions as defined in Yilmaz et al. (2017).

2. Materials and methods

2.1. Canola RBD oils

Harvested canola grain from multiple 2016 U.S. field trial locations was combined to form a pooled sample for each canola variety: LBFLFK, Kumily (the parental control canola variety for LBFLFK), and three other conventional canola varieties (IMC105, 46A65, and Wizzard). LBFLFK was treated in-field with the imidazolinone herbicide, imazamox. To minimize the chance of cross-pollination, the Kumily and conventional canola variety plots were located more than 1000 m apart from the LBFLFK plot. The canola grain was processed into RBD oil at Cargill, Incorporated (Fort Collins, CO) under commercial conditions using pilot-scale equipment. To maintain stability, mixed tocopherols (Cargill, Incorporated) were added to each canola RBD oil to achieve a final concentration of approximately 1000 ppm total tocopherols. All canola RBD oil samples were maintained frozen at −20 °C following production.

The Kumily and LBFLFK RBD oils used in the edible oils comparison study and in the 28-day repeated dose toxicity study were from the same production lot. The RBD oil produced from conventional canola varieties were used only in the edible oils comparison study.

2.2. Edible oils comparison study

2.2.1. Commercially-obtained fat and oil samples

Commercially-obtained edible oils and fat-containing foods were purchased from retail stores either online or in person. After purchase, these samples were maintained in frozen storage. Only products available for purchase and routinely consumed by humans in the U.S. were included in the study. In total, 31 edible oils and fat-containing foods were analyzed. A product category was assigned for the purpose of determining the appropriate analytical method. The complete list of products and product categories analyzed is detailed in Table 1.

All samples, including RBD canola oils, were shipped and stored frozen (−20 °C) until they were used for analysis. Oil and fish samples were nitrogen-capped before freezing. Prior to analysis, samples were thawed and prepared as needed according to the established methods for each product category.

2.2.2. Oil and food compositional analysis

All compositional analyses were performed at Eurofins Nutritional Analysis Center (ENAC; Des Moines, IA). Crude fat content for Meat and Fish category samples was determined by solvent extraction (reference method AOAC 960.39) and in all other samples by acid hydrolysis (reference methods AOAC 945.02, AOAC 989.05, AOAC 925.32, and AOAC 933.05). Fatty acid amounts were quantified by gas chromatography of fatty acid methyl ester derivatives (reference methods AOAC 996.06, AOCS Ce 2–66, AOCS Ce 1b-89, and AOCS Ce 1–62). The fatty acids measured were selected based on what is known to be present in the various edible oils and fat-containing foods, the fatty acids that are known to occur in conventional canola varieties (OECD, 2011), and the fatty acids that might be present in LBFLFK RBD oil based on substrate specificities of the introduced enzymes (Yilmaz et al., 2017).

Fatty acid nomenclature is used such that “Cx:y n-z” specifies a fatty acid with x carbon atoms and y double bonds, where the last double bond is located at the n-z position relative to the methyl end of the fatty acid. The fatty acids measured were C4:0, C6:0, C8:0, C10:0, C11:0, C12:0, C14:0, C14:1, C15:0, C15:1, C16:0, C16:1n-7, C16:1n-9, C16:1 trans, total C16:1, C16:3n 3, C17:0, C17:1, C18:0, C18:1n-7, C18:1n-9, C18:1 trans, total C18:1, C18:2n-6, C18:2n-9, C18:2 trans, total C18:2, C18:3n-3, C18:3n-6, C18:3 trans, total C18:3, C18:4n-3, total C18:4, C20:0, C20:1n-9, C20:2n-6, C20:2n-9, C20:3n-3, C20:3n-6, C20:3n-9, total C20:3, C20:4n-3, C20:4n-6, total C20:4, C20:5n-3 (EPA), C22:0, C22:1n-9, total C22:1, C22:2n-6, C22:4n-3, C22:4n-6, C22:5n-3, C22:5n-6, total C22:5, C22:6n-3 (DHA), C24:0, C24:1n-9, total C24:1, and total trans fatty acids. All measurements were performed in technical triplicate. Samples that were not homogenous in nature (e.g.,

Table 1
Edible oils and fat-containing foods for compositional analysis.

Product Category	Product Study ID	Product Purchased/Ordered
Dairy – Butter	Goat butter	Delamere Dairy brand goat butter
Dairy – Butter	Grass-fed cow butter	Kerry Gold brand unsalted butter
Dairy – Butter	Generic butter	Land O'Lakes brand unsalted butter
Dairy – Cheese	Goat cheese	Montchevre brand goat cheese
Dairy – Cheese	Blue cheese	Treasure Cave brand crumbled blue cheese
Dairy – Cheese	Other cheese	El Cortijo brand Manchego cheese
Egg	Generic eggs	Simple Truth brand grain-fed eggs, brown large
Egg	PUFA-rich eggs	Simple Truth brand omega-3 eggs, brown large
Fish	Wild salmon	Slanker Grass-Fed Meat brand sockeye salmon boneless fillet (Alaska wild-caught)
Fish	Salmon aquaculture (Norway)	Walmart brand Chilean salmon
Fish	Pangasius (swai filet)	Walmart brand frozen Swai fillet
Fish	Mackerel	King Mackerel fillets (North Carolina wild-caught)
Fish	Squid	Bos'n brand frozen Loligo squid
Fish	Anchovy	Beach Cliff brand sardines in water
Fish Oil	Algal oil 1	Martek brand Life's DHA 200 mg all-vegetarian softgels
Fish Oil	Algal oil 2	Ovega-3 brand vegetarian softgels
Fish Oil	Algal oil 3	Nordic Naturals brand algae omega, softgels
Fish Oil	Cod liver oil	Dropi brand pure Icelandic extra virgin cod liver oil
Fish Oil	Fish oil 1	Nature's Answer brand liquid omega-3 deep sea fish oil
Fish Oil	Menhaden oil	VWR International brand fish oil from menhaden
Fish Oil	Fish oil 2	Nature Made brand fish oil (1200 mg, 360 mg omega-3 in TAG form)
Fish Oil	Salmon oil	Spring Valley brand salmon oil
Meat	Grass-fed beef muscle 1	Certified Piedmonteset brand stew meat
Meat	Grass-fed calf liver	Slanker Grass-Fed Meat brand beef liver
Meat	Grass-fed beef muscle 2	Slanker Grass-Fed Meat brand ground beef, high fat
Meat	PUFA-rich chicken liver	Slanker Grass-Fed Meat brand omega-3 chicken livers
Meat	Corn-fed beef muscle	Walmart brand ground beef
Meat	Corn-fed chicken liver	Sanderson Farms brand chicken livers
Meat	Corn-fed beef liver	SkyLark brand calf liver
Meat – Other	Corn-fed beef marrow bones	Rumba Meats brand beef soup bones
Other Oils	*Mortierella alpina* oil	Molecular Nutrition brand X-Factor Advanced (arachidonic acid; 825 mg anabolic formula)

eggs, beef muscle, and salmon fillet) were homogenized and divided into aliquot amounts to use in technically replicated analysis.

2.2.3. Statistical analysis

The total fatty acid content of each sample was used together with the absolute amounts of individual fatty acids to calculate relative fatty acid content (% of total fatty acids). Relative fatty acid content allowed for easier comparison of samples with different fat content and consequently, relative fatty acid content was used for comparison purposes.

For calculating the mean of crude fat and absolute fatty acid amounts, all values below the limit of quantitation (LOQ) were set to 0.5*LOQ. For calculating the mean of relative fatty acid content, each replicate < LOQ was assigned a value equal to 0.5 the LOQ (% fresh weight (FW)) converted to LOQ (% relative), which was calculated as follows:

LOQ (% Relative) = (LOQ [% FW] / FA Total)

where LOQ (% FW) equals 0.02% for all fatty acids except for C16:1n-7 (0.04%) and C18:1n-7 (0.03%). "FA Total" refers to the total fatty acid content (% FW) for a given replicate. If a calculated mean was < LOQ (% relative), it was reported as " < LOQ," and no standard deviation was reported.

For "Total Saturated FA," "Total Monounsaturated FA," "Total Omega-6," and "Total Omega-3," the means of the appropriate individual fatty acids were summed, and no standard deviation was calculated.

Principal component analysis was performed using the prcomp

Table 2
Relative fatty acid profile of canola RBD oil samples.

	LBFLFK	Kumily	IMC105	46A65	Wizzard
C4:0	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
C6:0	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
C8:0	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
C10:0	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
C11:0	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
C12:0	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
C14:0	0.063 ± 0.002	0.056 ± 0.002	0.049 ± 0.002	0.041 ± 0.001	0.055 ± 0.001
C14:1	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
C15:0	0.042 ± 0.003	0.022 ± 0.001	< LOQ	< LOQ	< LOQ
C15:1	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
C16:0	4.669 ± 0.006	4.382 ± 0.007	4.033 ± 0.005	3.554 ± 0.005	3.884 ± 0.004
C16:1n-7	0.171 ± 0.001	0.238 ± 0.001	0.206 ± 0.001	0.188 ± 0.003	0.198 ± 0.001
C16:1n-9	0.046 ± 0.002	0.039 ± 0.001	0.044 ± 0.002	0.04 ± 0.003	0.037 ± 0.002
C16:1 trans	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
C16:3n-3	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
C17:0	0.053 ± 0.001	0.046 ± 0.002	0.044 ± 0.001	0.045 ± 0.002	0.039 ± 0.003
C17:1	0.027 ± 0.001	0.058 ± 0.002	0.058 ± 0	0.057 ± 0.004	0.053 ± 0
C18:0	3.108 ± 0.01	2.287 ± 0.01	2.063 ± 0.002	2.086 ± 0.001	2.166 ± 0.001
C18:1n-7	3.267 ± 0.006	3.18 ± 0.005	2.987 ± 0.003	2.908 ± 0.005	2.741 ± 0.003
C18:1n-9	30.397 ± 0.036	59.419 ± 0.02	61.424 ± 0.024	62.879 0.002	62 ± 0.023
C18:1 trans	0.073 ± 0.012	0.035 ± 0.007	0.04 ± 0.008	0.04 ± 0.009	0.032 ± 0.007
C18:2n-6	29.261 ± 0.034	19.45 ± 0.003	23.306 ± 0.01	18.282 ± 0.002	18.657 ± 0.009
C18:2n-9	1.416 ± 0.003	0.064 ± 0.002	0.067 ± 0.003	0.061 ± 0.001	0.057 ± 0.002
C18:2 trans	0.036 ± 0.005	< LOQ	< LOQ	< LOQ	< LOQ
C18:3n-3	4.875 ± 0.005	7.347 ± 0.011	2.527 ± 0.002	6.32 ± 0.005	6.834 ± 0.003
C18:3n-6	2.158 ± 0.004	< LOQ	< LOQ	< LOQ	< LOQ
C18:3 trans	0.509 ± 0.009	0.591 ± 0.001	0.21 ± 0.001	0.524 ± 0.002	0.472 ± 0.003
C18:4n-3	0.308 ± 0.002	< LOQ	< LOQ	< LOQ	< LOQ
C20:0	0.684 ± 0.001	0.706 ± 0.002	0.685 ± 0.001	0.683 ± 0.001	0.648 ± 0.002
C20:1n-9	0.8 ± 0.003	1.121 ± 0.002	1.219 ± 0.001	1.282 ± 0.007	1.114 ± 0.002
C20:2n-6	0.107 ± 0.002	0.055 ± 0.001	0.065 ± 0.001	0.061 ± 0.001	0.056 ± 0.001
C20:2n-9	0.36 ± 0.003	< LOQ	< LOQ	< LOQ	< LOQ
C20:3n-3	0.058 ± 0.002	< LOQ	< LOQ	< LOQ	< LOQ
C20:3n-6	4.899 ± 0.083	< LOQ	< LOQ	< LOQ	< LOQ
C20:3n-9	0.048 ± 0.001	< LOQ	< LOQ	< LOQ	< LOQ
C20:4n-3	1.895 ± 0.002	< LOQ	< LOQ	< LOQ	< LOQ
C20:4n-6	1.729 ± 0.002	< LOQ	< LOQ	< LOQ	< LOQ
C20:5n-3	3.972 ± 0.021	< LOQ	< LOQ	< LOQ	< LOQ
C22:0	0.275 ± 0.003	0.345 ± 0.001	0.369 ± 0.001	0.352 ± 0.001	0.312 ± 0.001
C22:1n-9	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
C22:2n-6	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
C22:4n-3	0.983 ± 0.004	< LOQ	< LOQ	< LOQ	< LOQ
C22:4n-6	0.499 ± 0.004	< LOQ	< LOQ	< LOQ	< LOQ
C22:5n-3	2.14 ± 0.008	< LOQ	< LOQ	< LOQ	< LOQ
C22:5n-6	0.046 ± 0.005	< LOQ	< LOQ	< LOQ	< LOQ
C22:6n-3	0.346 ± 0.003	< LOQ	< LOQ	< LOQ	< LOQ
C24:0	0.241 ± 0.003	0.189 ± 0.002	0.218 ± 0.001	0.19 ± 0.001	0.247 ± 0.001
C24:1n-9	0.094 ± 0.002	0.131 ± 0.001	0.146 ± 0.001	0.132 ± 0.001	0.138 ± 0.001
Total Trans FA	0.617 ± 0.025	0.626 ± 0.006	0.251 ± 0.008	0.565 ± 0.009	0.505 ± 0.01
Total Saturated FA[a]	9.135	8.033	7.462	6.951	7.352
Total Mono-unsaturated FA[a]	34.802	64.186	66.085	67.485	66.280
Total Omega-6 FA[a]	38.697	19.505	23.371	18.343	18.713
Total Omega-3 FA[a]	14.576	7.347	2.527	6.320	6.834

LOQ = limit of quantitation.
Data are the mean % of total fatty acids ± SD.
[a] Values are the sum of means and, therefore, ± SD is not provided.

function in R (R Core Team, 2016) with the parameters center = TRUE and scale = TRUE. The data used for principal component analysis included the mean relative fatty acid amounts of all 48 individual fatty acids across all edible oils and fat-containing foods, including LBFLFK RBD oil and conventional canola varieties.

2.3. 28-Day repeated dose toxicity study

2.3.1. Experimental design

LBFLFK RBD oil was administered by oral gavage at two dose levels: 1) a low dose, a mixture of LBFLFK RBD oil (approximately 33%) and Kumily RBD oil (approximately 67%), and 2) a high dose of undiluted LBFLFK RBD oil (100%). Kumily RBD oil served as a control substance and drinking water served as an additional control substance (data not shown). Commercially available menhaden oil with natural tocopherols (OmegaPure E) was provided by Bioriginal (Irvine, CA). Because certain clinical pathology endpoints can be influenced in rats by overall LC-PUFA consumption (Hempenius et al., 2000; Lina et al., 2006; Blum et al., 2007; Kawashima et al., 2009), menhaden oil was used as a reference substance. Because the fatty acid profile and LC-PUFA content of LBFLFK RBD oil and menhaden oil are similar, menhaden oil served as a relevant comparison for determining the biological relevance of any statistically significant differences found between LBFLFK and Kumily RBD oils.

All substances were administered by oral gavage (3 mL (or 2.76 g)/kg body weight/day (g/kg bw/d)) to male and female Wistar rats for four weeks. This dose selection is consistent with the safety assessments of other LC PUFA-containing oils when administered by oral gavage in a repeated-dose toxicity study (Hammond et al., 2008; Kawashima et al., 2009; Belcher et al., 2011). The limit dose for a 28-day repeated dose toxicity study in rodents is 1000 mg/kg bw/d (OECD, 2008). LBFLFK RBD oil at high dose contains at least 45% total LC-PUFAs. Thus, 45% of 2.76 g/kg bw/d is 1.24 g/kg bw/d, which exceeds the recommended limit dose for a 28-day repeated dose toxicity study. For EPA and DHA specifically, LBFLFK RBD oil at high dose contains at least 5% of these combined fatty acids. At 2.76 g/kg bw/d, the dose of EPA and DHA is 0.138 g/kg bw/d, which corresponds to an approximately 3-fold increase over the FDA approved daily intake of EPA and DHA from menhaden oil based on human safety information (3 g/60 kg (adult human)/d = 0.05 g/kg bw/d) (U.S. FDA, 1999).

LBFLFK and Kumily RBD oils were characterized by compositional analyses at ENAC using accepted AOAC International or other recognized methods. The following parameters were measured: fatty acid profile, phytosterols, arsenic, cadmium, lead, mercury, peroxide value, p-Anisidine value, vitamin K1, and tocopherols. A pesticide residue panel was also performed at Covance Laboratories, Inc. (Greenfield, IN). Because of the presence of LC-PUFAs, particularly EPA and DHA, in LBFLFK RBD oil, it was possible to distinguish Kumily and LBFLFK RBD oils based on their fatty acid profiles (for example, see C20:5n-3 and C22:6n-3 in Table 2). The stability of the Kumily and LBFLFK RBD oils under storage and use conditions was demonstrated by compositional analyses at ENAC by analyzing fatty acids, peroxide value, and p-Anisidine value.

2.3.2. Animals

The study was conducted at BASF Experimental Toxicology and Ecology Laboratories in Ludwigshafen, Germany in accordance with the Organization for Economic Co-operation and Development (OECD) Principles of Good Laboratory Practice (GLP) and the GLP principles of the German Chemicals Act. The study was conducted according to OECD Guidelines Method No. 407 for repeated dose 28-day oral toxicity study in rodents (OECD, 2008). The performing laboratory is certified by the Association for Assessment and Accreditation of Laboratory Animal Care and acted in accordance with the German Animal Welfare Act and the relevant European Council Directive. The study was notified to the local authority under approval number 23 177–07.

Male and female Wistar rats were obtained from Charles River Laboratories (Sulzfeld, Germany). At the start of the administration period, the animals were approximately 42 days old with mean weights of 176 g (males) and 124 g (females). Animals were distributed according to weight among the individual treatment groups with 20 rats in each group (ten males and ten females) for a total of 100 rats. The number of animals used per group was based on the recommendation by EFSA for risk assessment of food and feed from genetically modified plants (EFSA Panel on Genetically Modified Organisms, 2011). Animals were separated into treatment groups by randomization such that the mean body weight across groups did not vary by more than 20%. Rats were group-housed for the entire study period (five animals per cage). Animals were acclimated to the laboratory environment for nine days prior to study initiation. The animal rooms were maintained at a relative humidity of 30–70%, room temperature of 20–24 °C, and 12 h light/12 h dark cycle. Animals were provided *ad libitum* access to feed, standard rat maintenance diet (ground Kliba mouse/rat maintenance diet "GLP" meal, Provimi Kliba SA, Kaiseraugst, Switzerland), and drinking water.

2.3.3. Clinical observations, body weight change, and feed consumption

Health status and cage-side clinical signs were checked at least once daily while body weight and feed consumption were measured weekly. Detailed clinical observations (e.g., abnormal response to handling, evaluations of fur, skin, eyes, and mucous membranes) were recorded weekly. At the end of the administration period, a function observation battery, including home cage-side, open field observations, and sensory motor and reflex tests, as well as measurement of motor activity, was performed on all animals.

2.3.4. Clinical pathology

Animals were fasted for at least 16 h prior to collection of blood on study day 29 just before necropsy. The following hematological indices were evaluated from the extracted blood using an ADVIA 120 analyzer (Siemens, Munich, Germany): red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), reticulocytes (RETA), blood platelet count (PLT), prothrombin time (Hepato Quick's test, HQT), white blood cells (WBC), differential blood counts (polymorphonuclear neutrophils absolute (NEUTA), lymphocytes absolute (LYMPHA), monocytes absolute (MONOA), eosinophils absolute (EOSA), and basophils absolute (BASOA).

The clinical variables evaluated from serum were measured on a COBAS c501 analyzer (Roche, Mannheim, Germany) and the parameters included alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), total protein (TPROT), albumin (ALB), creatinine (CREA), glucose (GLUC), calcium (CA), inorganic phosphate (INP), cholesterol (CHOL), triglycerides (TRIG), urea (UREA), total bilirubin (TBIL), globulins (GLOB), total bile acids (TBA), sodium (NA), potassium (K), and chloride (CL).

On the afternoon before urinalysis, the animals were transferred individually into metabolism cages with no food or drinking water provided. The following parameters were evaluated during urinalysis: volume, color, turbidity, urine dipstick parameters (pH value, protein, glucose, ketones, urobilinogen, bilirubin, and blood), specific gravity (refractometry), and microscopy of sediment.

2.3.5. Gross necropsy and histopathology

The rats were sacrificed by decapitation following anesthesia and a general necropsy evaluation was performed on the exsanguinated animals to detect gross lesions. The following organs were weighed and examined: adrenal glands, brain, epididymides, heart, kidneys, liver, ovaries, prostate, seminal vesicles with coagulating gland, spleen, testes, thymus, thyroid glands, and uterus with cervix. The organs were

then fixed and processed for histopathological evaluation.

2.3.6. Statistical analysis

The statistical analyses were performed using Kumily RBD oil as the control substance. LBFLFK RBD oil at both low and high dose were compared to Kumily RBD oil. If statistically significant differences between LBFLFK RBD oil at either low or high dose were found compared to Kumily RBD oil, then that dose of LBFLFK RBD oil was compared to menhaden oil. Menhaden oil was not compared to LBFLFK RBD oil in instances where LBFLFK RBD oil was not statistically different from Kumily RBD oil. Menhaden oil was also not directly compared to Kumily RBD oil for any analysis. For all measurements, mean and standard deviation (SD) were calculated. For the measurement of body weight and body weight changes, a Dunnett's *t*-test was performed. For all other parameters, non-parametric one-way analysis using KRUSKAL-WALLIS test was applied. If the resulting *p*-value was equal or less than 0.05, a pairwise comparison of each test group with the control group was performed using WILCOXON-test (two-sided) for the hypothesis of equal medians. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Edible oils comparison study

3.1.1. Fatty acid profile of LBFLFK RBD oil compared to other canola oils

The relative fatty acid profiles of canola oil samples, including RBD oil samples from LBFLFK, Kumily (parental control canola variety for LBFLFK), and three conventional canola varieties, are provided in Table 2. Canola oil by definition has a low content of saturated fatty acids, a high content of monounsaturated fatty acids, and a low content (< 2% of total fatty acids) of erucic acid (C22:1n-9) (OECD, 2011). LBFLFK RBD oil has features that are typical of canola oil, namely low total saturated fat (~9%) and very low erucic acid (< LOQ). However, total monounsaturated fatty acids in LBFLFK RBD oil (~35%) are only about half of what is contained in the conventional canola varieties tested (~66%), driven largely by a decrease in C18:1n-9. The amount of C18:2n-6 is roughly 1.5-fold higher in LBFLFK RBD oil than in RBD oils from Kumily and the conventional canola varieties. The changes to the amounts of C18:1n-9 and C18:2n-6, both of which are normally present in conventional canola varieties, can be explained by the fact that C18:1n-9 is the starting substrate and C18:2n-6 is the first product of the introduced biosynthetic pathway (Fig. 1). The introduction of the enzymatic pathway in LBFLFK also results in the presence of fatty acids not normally found in conventional canola varieties. Namely, the fatty acids C18:3n-6, C18:4n-3, C20:2n-9, C20:3n-3, C20:3n-6, C20:3n-9, C20:4n-3, C20:4n-6, C20:5n-3, C22:4n-3, C22:4n-6, C22:5n-3, C22:5n-6, and C22:6n-3 were consistently below LOQ in RBD oils from Kumily and conventional canola varieties but were present in LBFLFK RBD oil. As a result, the total omega-6 and total omega-3 fatty acid content are both 2-fold higher in LBFLFK RBD oil than in the other varieties. Most of the fatty acids found in LBFLFK RBD oil but not in other canola RBD oils are intermediates of the EPA and DHA biosynthetic pathway (see Fig. 1). However, four fatty acids (C20:2n-9, C20:3n-9, C20:3n-3, and C22:4n-3) result from minor side reactions of the introduced enzymes (Yilmaz et al., 2017) and are not converted to EPA or DHA (see Fig. 1).

3.1.2. Fatty acid profile of LBFLFK RBD oil compared to edible oils and fat-containing foods

The relative amounts of all fatty acids measured in the edible oils and fat-containing foods are provided as Supplementary Table 1. Principal component analysis was performed using the relative amounts of all individual fatty acids to investigate the relationship between the samples (Fig. 2). A total of 36 principal components (PC) were identified, 13 of which could explain 95% of the variance (Fig. 2A). Fig. 2B shows a plot of PC1 and PC2, which individually account for 25% and 17% of the variance, respectively. The Euclidian distance between two samples is an indication of their similarity based on fatty acid profile. This analysis showed that LBFLFK RBD oil is most closely related to the oil from *Mortierella alpina*, the conventional canola variety oils, and wild and aquaculture salmon.

An additional comparison of LBFLFK RBD oil with the commercially available edible oils and fat-containing foods was performed by assessing the presence or absence (above or below LOQ) of individual fatty acids. All fatty acids present in LBFLFK RBD oil could be detected in at least one other sample. Based on the presence/absence of individual fatty acids, the samples most similar to LBFLFK RBD oil were the Menhaden oil, fish oils, salmon oil, and *Mortierella alpina* oil, all of which have GRAS status with the U.S. FDA (U.S. FDA, 1999, 2001, 2002, 2004b, 2017). The fatty acid profile of LBFLFK RBD oil compared

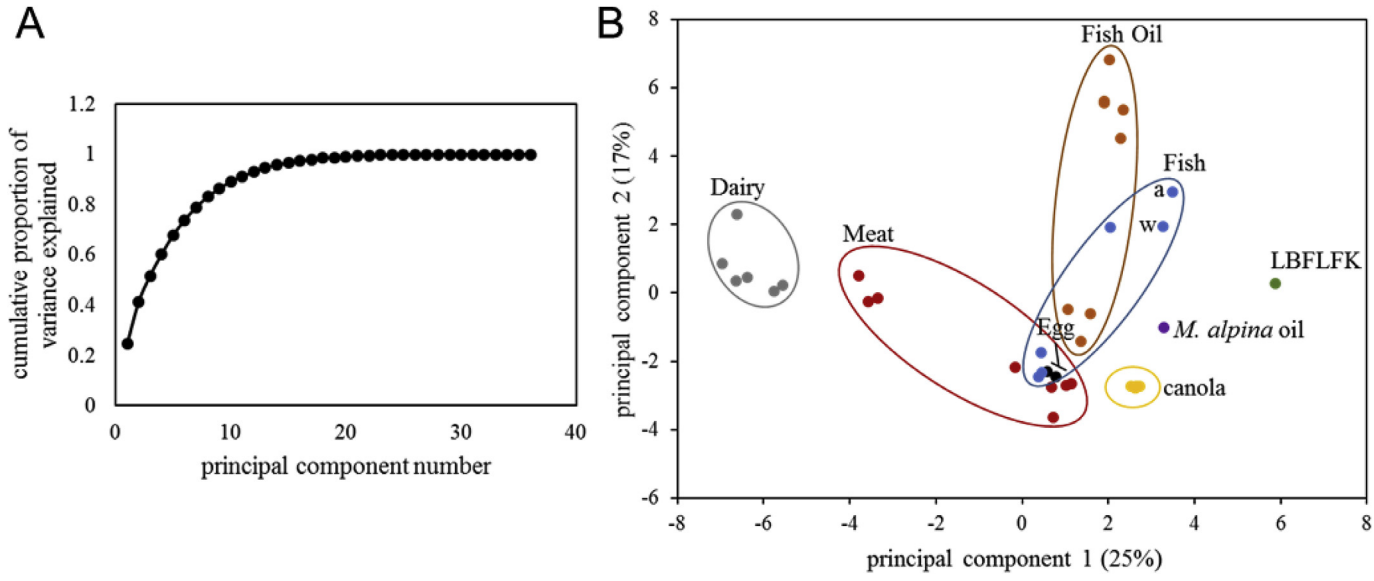

Fig. 2. Principal component analysis of the relative fatty acid amounts in all edible oils and fat-containing food samples. A) Cumulative proportion of variance as a function of the number of principal components (PC). B) All edible oils and fat-containing foods, including RBD oils from LBFLFK and conventional canola varieties, plotted as a function of PC1 and PC2, which account for 25% and 17% of variance, respectively. “a” and “w” indicate aquaculture and wild salmon samples, respectively.

to fish oils and *Mortierella alpina* oil are presented in Table 3. Compared to *Mortierella alpina* oil, LBFLFK RBD oil contained three additional fatty acids (C20:3n-3, C20:3n-9, and C22:4n-3) and lacked three other ones (C12:0, C16:1 trans, and C16:3n-3). The menhaden oil, fish oils, and salmon oil contained six fatty acids that were not present in LBFLFK RBD oil but were otherwise similar. Other differences between LBFLFK RBD oil and the menhaden oil, fish oils, and salmon oil were that LBFLFK RBD oil has lower total saturated and total omega-3 fatty acids but higher total omega-6 fatty acids. Six fatty acids (C18:2n-6, C18:2n-9, C18:3n-6, C20:2n-9, C20:4n-3, and C22:4n-3) were identified that had the highest relative abundance in LBFLFK RBD oil compared to the other samples included in this study.

3.2. 28-Day repeated dose toxicity study

3.2.1. Influences on rat clinical observations, body weight, and feed consumption

No deaths or overt signs of toxicity were observed during the course of the study. All animals appeared healthy and no adverse clinical observations were reported. No statistically significant differences were reported in mean weekly body weight change (Table 4) or in mean body weights (Fig. 3) for males when comparing LBFLFK RBD oil at low or high dose to Kumily RBD oil. In females, body weight change was consistently and statistically significantly higher in LBFLFK RBD oil at low and high dose versus Kumily RBD oil. However, since none of the body weight changes in females were statistically significantly different from menhaden oil and no statistically significant differences were

Table 3
Relative fatty acid profile of LBFLFK RBD oil compared to fish and other oils.

Product Sample	LBFLFK	Fish Oil 1	Menhaden Oil	Fish Oil 2	Salmon Oil	*Mortierella alpina* Oil
C4:0	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
C6:0	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
C8:0	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
C10:0	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
C11:0	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
C12:0	< LOQ	0.123 ± 0.007	0.125 ± 0.002	0.125 ± 0.004	0.073 ± 0.006	0.055 ± 0.003
C14:0	0.063 ± 0.002	7.35 ± 0.037	8.576 ± 0.003	7.125 ± 0.013	4.571 ± 0.006	2.515 ± 0.076
C14:1	< LOQ	0.038 ± 0.002	0.061 ± 0.003	0.032 ± 0.001	0.031 ± 0.003	< LOQ
C15:0	0.042 ± 0.003	0.509 ± 0.01	0.694 ± 0.003	0.525 ± 0.01	0.466 ± 0.006	0.219 ± 0.011
C15:1	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
C16:0	4.669 ± 0.006	16.099 ± 0.048	16.6 ± 0.029	16.521 ± 0.02	13.716 ± 0.018	10.38 ± 0.266
C16:1n-7	0.171 ± 0.001	8.029 ± 0.044	11.806 ± 0.022	8.236 ± 0.025	5.512 ± 0.068	2.482 ± 0.07
C16:1n-9	0.046 ± 0.002	0.377 ± 0.001	0.325 ± 0	0.375 ± 0.023	0.31 ± 0.01	0.121 ± 0.01
C16:1 trans	< LOQ	0.644 ± 0.024	0.693 ± 0.014	0.62 ± 0.008	0.512 ± 0.007	0.186 ± 0.009
C16:3 n-3	< LOQ	1.501 ± 0.034	1.581 ± 0.005	1.375 ± 0.023	0.601 ± 0.021	0.397 ± 0.01
C17:0	0.053 ± 0.001	0.452 ± 0.009	0.649 ± 0.208	0.49 ± 0.009	0.462 ± 0.01	0.292 ± 0.012
C17:1	0.027 ± 0.001	0.15 ± 0.004	0.182 ± 0.007	0.177 ± 0.013	0.246 ± 0.005	0.057 ± 0.001
C18:0	3.108 ± 0.01	3.234 ± 0.029	2.889 ± 0.008	3.445 ± 0.016	3.245 ± 0.01	5.905 ± 0.11
C18:1n-7	3.267 ± 0.006	2.928 ± 0.017	3.144 ± 0.009	3.076 ± 0.006	2.911 ± 0.022	1.02 ± 0.017
C18:1n-9	30.397 ± 0.036	8.526 ± 0.03	6.138 ± 0.008	8.241 ± 0.026	20.779 ± 0.067	6.994 ± 0.117
C18:1 trans	0.073 ± 0.012	0.267 ± 0.106	0.172 ± 0.029	0.295 ± 0.137	0.396 ± 0.205	0.115 ± 0.017
C18:2n-6	29.261 ± 0.034	1.193 ± 0.002	1.363 ± 0.001	1.145 ± 0.008	5.801 ± 0.014	6.962 ± 0.139
C18:2n-9	1.416 ± 0.003	0.165 ± 0.003	0.101 ± 0.003	0.164 ± 0.003	0.11 ± 0.005	0.158 ± 0.005
C18:2 trans	0.036 ± 0.005	0.191 ± 0.053	0.316 ± 0.062	0.224 ± 0.063	0.162 ± 0.067	0.099 ± 0.091
C18:3n-3	4.875 ± 0.005	0.766 ± 0.008	1.236 ± 0.008	0.67 ± 0.007	2.346 ± 0.001	0.245 ± 0.005
C18:3n-6	2.158 ± 0.004	0.232 ± 0.006	0.319 ± 0.009	0.228 ± 0.003	0.168 ± 0.009	1.778 ± 0.032
C18:3 trans	0.509 ± 0.009	0.238 ± 0.011	0.525 ± 0.002	0.348 ± 0.009	0.359 ± 0.016	0.092 ± 0.006
C18:4n-3	0.308 ± 0.002	2.787 ± 0.01	2.973 ± 0.005	2.373 ± 0.014	1.605 ± 0.016	0.783 ± 0.008
C20:0	0.684 ± 0.001	0.214 ± 0.005	0.225 ± 0.007	0.29 ± 0.018	0.297 ± 0	0.69 ± 0.018
C20:1n-9	0.8 ± 0.003	1.018 ± 0.006	0.958 ± 0.005	1.304 ± 0.005	2.331 ± 0.013	0.442 ± 0.007
C20:2n-6	0.107 ± 0.002	0.19 ± 0.01	0.24 ± 0.004	0.177 ± 0.002	0.595 ± 0.01	0.348 ± 0.006
C20:2n-9	0.36 ± 0.003	0.111 ± 0.003	0.129 ± 0.008	0.127 ± 0.005	0.064 ± 0.003	0.046 ± 0.022
C20:3n-3	0.058 ± 0.002	0.181 ± 0.003	0.255 ± 0.008	0.185 ± 0.011	0.321 ± 0.009	< LOQ
C20:3n-6	4.899 ± 0.083	0.158 ± 0.008	0.242 ± 0.002	0.179 ± 0.003	0.179 ± 0.007	3.077 ± 0.031
C20:3n-9	0.048 ± 0.001	0.059 ± 0.01	0.039 ± 0.004	0.054 ± 0.006	0.056 ± 0.005	< LOQ
C20:4n-3	1.895 ± 0.002	0.749 ± 0.01	1.523 ± 0.004	0.76 ± 0	0.699 ± 0.017	0.207 ± 0.004
C20:4n-6	1.729 ± 0.002	1.129 ± 0.006	1.133 ± 0.006	1.374 ± 0.006	0.993 ± 0.009	33.117 ± 0.586
C20:5n-3	3.972 ± 0.021	17.514 ± 0.037	14.561 ± 0.036	17.241 ± 0.031	9.696 ± 0.041	4.849 ± 0.084
C22:0	0.275 ± 0.003	0.121 ± 0.007	0.155 ± 0.008	0.151 ± 0.016	0.144 ± 0.006	1.724 ± 1.476
C22:1n-9	< LOQ	0.147 ± 0.007	0.164 ± 0.005	0.206 ± 0.016	0.31 ± 0.015	< LOQ
C22:2n-6	< LOQ	0.064 ± 0.003	0.084 ± 0.001	0.075 ± 0.008	0.088 ± 0.011	< LOQ
C22:4n-3	0.983 ± 0.004	0.079 ± 0.002	0.109 ± 0.007	0.075 ± 0.005	0.049 ± 0.004	< LOQ
C22:4n-6	0.499 ± 0.004	0.098 ± 0.003	0.218 ± 0.002	0.133 ± 0.007	0.106 ± 0.002	0.261 ± 0.011
C22:5n-3	2.14 ± 0.008	1.959 ± 0.009	2.485 ± 0.007	2.145 ± 0.012	1.486 ± 0.023	0.539 ± 0.009
C22:5n-6	0.046 ± 0.005	0.464 ± 0.003	0.446 ± 0.009	0.475 ± 0.002	0.53 ± 0.023	0.102 ± 0.004
C22:6n-3	0.346 ± 0.003	13.165 ± 0.04	11.408 ± 0.026	11.848 ± 0.017	12.564 ± 0.037	3.225 ± 0.054
C24:0	0.241 ± 0.003	0.067 ± 0.006	0.115 ± 0.006	0.109 ± 0.007	0.093 ± 0.004	8.278 ± 0.135
C24:1n-9	0.094 ± 0.002	0.477 ± 0.018	0.304 ± 0.004	0.459 ± 0.016	0.433 ± 0.006	0.345 ± 0.011
Total Trans FA	0.617 ± 0.025	1.339 ± 0.089	1.707 ± 0.08	1.487 ± 0.08	1.429 ± 0.119	0.492 ± 0.076
Total Saturated FA[a]	9.135	28.170	30.028	28.781	23.069	30.058
Total Mono-unsaturated FA[a]	34.802	21.690	23.082	22.108	32.862	11.515
Total Omega-6 FA[a]	38.697	3.527	4.044	3.785	8.462	45.645
Total Omega-3 FA[a]	14.576	38.701	36.131	36.673	29.368	9.848

LOQ = limit of quantitation.
Data are the mean % of total fatty acids ± SD.
[a] Values are the sum of means and, therefore, ± SD is not provided.

Table 4
Mean weekly body weight (g) change.

	Kumily RBD oil	LBFLFK RBD oil (low)	LBFLFK RBD oil (high)	Menhaden oil
Males				
Days 0–7	44.0 ± 3.6	45.2 ± 6.7	43.1 ± 4.8	43.6 ± 4.7
Days 0–14	78.2 ± 5.3	79.9 ± 11.9	70.6 ± 7.9	75.1 ± 8.8
Days 0–21	109.2 ± 8.7	105.9 ± 15.4	107.8 ± 12.4	109.6 ± 10.4
Days 0–28	125.8 ± 12.6	130.6 ± 18.2	129.2 ± 16.7	128.5 ± 13.5
Females				
Days 0–7	14.3 ± 2.3	19.6 ± 4.4[a]	21.9 ± 4.6[a]	18.1 ± 5.3
Days 0–14	29.2 ± 7.1	35.4 ± 9.0	37.0 ± 3.7[a]	32.1 ± 6.3
Days 0–21	41.8 ± 7.0	50.8 ± 5.7[a]	49.4 ± 6.2[a]	49.1 ± 3.9
Days 0–28	53.2 ± 6.8	60.6 ± 7.1[a]	57.4 ± 6.1	58.3 ± 2.3

Mean ± SD; n = 10 per group.
[a] = LBFLFK RBD oil was statistically significantly different ($p < 0.05$) from Kumily RBD oil. None of these statistically significant differences were statistically significantly different from menhaden oil. Menhaden oil was not compared to LBFLFK RBD oil in instances where LBFLFK RBD oil was not statistically different from Kumily RBD oil. Menhaden oil was also not directly compared to Kumily RBD oil.

observed in mean body weights for females when comparing LBFLFK RBD oil at low or high dose to Kumily RBD oil, these changes were considered unrelated to treatment and biologically not relevant.

Mean male and female feed consumption is presented in Fig. 4.

3.2.2. Clinical, functional and motor activity observations

No treatment-related effects were observed in home cage or open field observations or in sensory motor/reflex tests for males or females in LBFLFK RBD oil at low or high dose when compared to Kumily RBD oil. Likewise, no statistically significant differences in rearing, forelimb or hindlimb grip strength, or landing foot-splay were reported for either sex when comparing LBFLFK RBD oil at low or high dose to Kumily RBD oil. Regarding motor activity measurements, no statistically significant differences were observed for males when comparing LBFLFK RBD oil at low or high dose to Kumily RBD oil. For females, isolated changes in motor activity were seen at two middle intervals with both doses of LBFLFK RBD oil as compared to Kumily RBD oil. However, the values at these intervals were not statistically significantly different from menhaden oil, and this finding was concluded to be incidental and unrelated to treatment (data not shown).

3.2.3. Influences on rat hematology values

For hematology values (Table 5), no statistically significant differences were found in male rats when comparing LBFLFK RBD oil at low or high dose to Kumily RBD oil. For female rats, HGB, HCT, and MCV were statistically significantly lower when comparing LBFLFK RBD oil at high dose to Kumily RBD oil, but these values were not statistically significantly different from menhaden oil and all values were within the historical control range (Table 7). HCT was also statistically significantly lower when comparing LBFLFK RBD oil at low dose to Kumily RBD oil, but as with LBFLFK RBD oil at high dose, this change was not statistically significantly different from menhaden oil and was within the historical control range (Table 7). For MCH, LBFLFK RBD oil at high dose was statistically significantly lower compared to Kumily RBD oil and menhaden oil; however, all values for this parameter were within the historical control range (Table 7). Thus, these changes were not considered treatment-related or adverse.

3.2.4. Influences on rat serum chemistry values

For serum chemistry values (Table 6), TBIL was statistically significantly lower in male rats when comparing LBFLFK RBD oil at low and high dose to Kumily RBD oil. For female rats, GLUC was statistically significantly higher when comparing LBFLFK RBD oil at high dose to Kumily RBD oil. However, none of these changes were statistically significantly different from menhaden oil and all values were within the historical control range (Table 7). Thus, these changes were not considered treatment-related or adverse.

3.2.5. Urinalysis

For male rats, no statistically significant differences were found for urinalysis parameters when comparing LBFLFK RBD oil at low or high dose to Kumily RBD oil. For female rats, volume was statistically significantly higher and consequently, specific gravity was statistically significantly lower at low dose LBFLFK RBD oil versus Kumily RBD oil. Specific gravity was also statistically significantly lower at high dose LBFLFK RBD oil as compared to Kumily RBD oil. However, for both parameters, no dose-response relationship was observed, and no statistically significant differences were found when compared to menhaden oil (data not shown). These findings were concluded to be incidental and unrelated to treatment.

3.2.6. Influences on rat absolute organ weights

For absolute organ weights (Table 8), no statistically significant differences were found in male rats when comparing LBFLFK RBD oil at low or high dose to Kumily RBD oil. For female rats, absolute liver weight was statistically significantly higher when comparing LBFLFK RBD oil at high dose to Kumily RBD oil, but this value was not statistically significantly different from menhaden oil and was within the historical control range for absolute liver weights for females (means of 4.170–4.978 g). Thus, these changes were not considered treatment-related or adverse.

3.2.7. Influences on rat relative organ weights

For relative organ weights (Table 9), no statistically significant differences were found in male rats when comparing LBFLFK RBD oil at low or high dose to Kumily RBD oil. For female rats, relative heart weight was statistically significantly lower when comparing LBFLFK

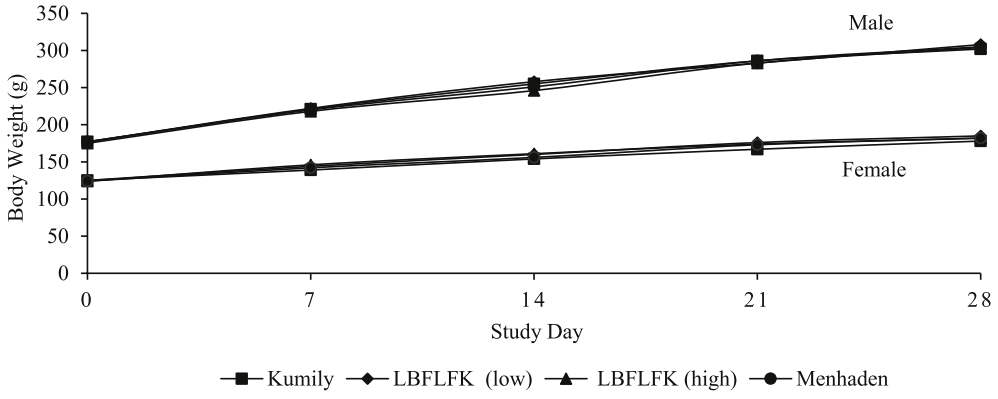

Fig. 3. Mean male and female body weights.

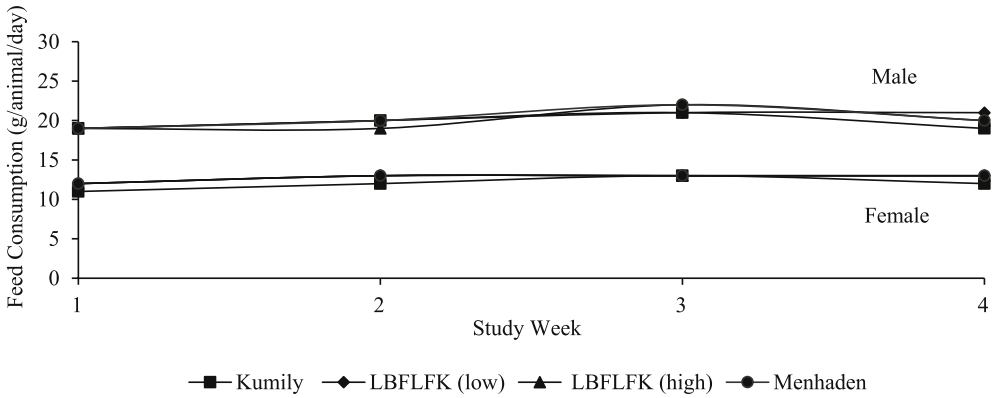

Fig. 4. Mean male and female feed consumption.

RBD oil at low and high dose to Kumily RBD oil, but these values were not statistically significantly different from menhaden oil and were within the historical control range for relative heart weight for females (means of 0.330–0.403%). Thus, these changes were not considered treatment-related or adverse.

3.2.8. Histopathology

Histopathology was performed on all animals (10 males and 10 females per group) in the Kumily and LBFLFK RBD oil groups. The microscopic findings are summarized in Table 10. All gross and microscopic events were either single observations or were distributed in equal incidence over control and treatment groups. Furthermore, all findings are well known background findings in rats (Boorman, 1990; McInnes, 2012; Suttie, 2018). Thus, these lesions were considered incidental or spontaneous in origin and not treatment-related.

4. Discussion

Canola RBD oil is food-grade oil that is not reported to have quantifiable levels of proteins, which is likely due to the heat and chemical treatments encountered during processing (Tattrie and Yaguchi, 1973; Crevel et al., 2000; Zitouni et al., 2000; Martín-Herná;ndez et al., 2008). None of the newly expressed proteins introduced by the biosynthetic pathway into LBFLFK were detectable in LBFLFK RBD oil (data not shown). The aim of this study was to evaluate the safety of the fatty acids contained in LBFLFK RBD oil.

Table 5
Rat hematology values for males and females.

	Kumily RBD oil	LBFLFK RBD oil (low)	LBFLFK RBD oil (high)	Menhaden oil
Males				
RBC (10^{12}/L)	7.72 ± 0.35	7.87 ± 0.28	7.59 ± 0.26	7.69 ± 0.21
HGB (mmol/L)	9.2 ± 0.3	9.4 ± 0.3	9.0 ± 0.2	9.1 ± 0.2
HCT (%)	0.416 ± 0.014	0.429 ± 0.017	0.411 ± 0.014	0.411 ± 0.010
MCV (fL)	53.9 ± 1.6	54.5 ± 1.9	54.1 ± 1.2	53.4 ± 1.7
MCH (fmol)	1.19 ± 0.04	1.19 ± 0.05	1.19 ± 0.03	1.18 ± 0.03
MCHC (mmol/L)	22.04 ± 0.55	21.86 ± 0.53	21.98 ± 0.40	22.08 ± 0.32
RETA (10^9/L)	139.0 ± 25.3	148.8 ± 28.9	151.5 ± 12.9	132.1 ± 21.5
PLT (10^9/L)	779 ± 133	753 ± 112	729 ± 137	698 ± 29
HQT (sec)	39.2 ± 2.2	38.5 ± 2.4	38.0 ± 0.6	41.2 ± 3.1
WBC (10^9/L)	7.24 ± 1.34	7.07 ± 0.84	6.80 ± 1.17	7.08 ± 1.59
NEUTA (10^9/L)	0.97 ± 0.15	0.90 ± 0.16	0.85 ± 0.16	0.95 ± 0.28
LYMPHA (10^9/L)	5.96 ± 1.38	5.88 ± 0.81	5.68 ± 1.04	5.87 ± 1.49
MONOA (10^9/L)	0.15 ± 0.03	0.14 ± 0.04	0.11 ± 0.03	0.12 ± 0.03
EOSA (10^9/L)	0.10 ± 0.02	0.11 ± 0.03	0.11 ± 0.05	0.10 ± 0.03
BASOA (10^9/L)	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
Females				
RBC (10^{12}/L)	7.47 ± 0.16	7.40 ± 0.18	7.41 ± 0.18	7.32 ± 0.32
HGB (mmol/L)	8.7 ± 0.2	8.5 ± 0.2	8.4 ± 0.2[a]	8.6 ± 0.3
HCT (%)	0.406 ± 0.010	0.394 ± 0.008[a]	0.393 ± 0.007[a]	0.397 ± 0.018
MCV (fL)	54.3 ± 1.4	53.3 ± 1.3	53.0 ± 0.8[a]	54.3 ± 1.7
MCH (fmol)	1.16 ± 0.03	1.15 ± 0.02	1.13 ± 0.02[a, b].	1.17 ± 0.04
MCHC (mmol/L)	21.41 ± 0.23	21.58 ± 0.35	21.41 ± 0.33	21.61 ± 0.36
RETA (10^9/L)	158.7 ± 32.2	179.4 ± 59.6	194.3 ± 36.8	136.8 ± 29.3
PLT (10^9/L)	742 ± 92	791 ± 57	780 ± 54	697 ± 84
HQT (sec)	36.9 ± 1.9	35.2 ± 2.7	35.7 ± 1.5	38.0 ± 1.7
WBC (10^9/L)	4.72 ± 0.73	5.18 ± 1.31	4.20 ± 0.90	4.09 ± 1.23
NEUTA (10^9/L)	0.54 ± 0.11	0.60 ± 0.20	0.61 ± 0.15	0.53 ± 0.08
LYMPHA (10^9/L)	4.01 ± 0.66	4.41 ± 1.27	3.44 ± 0.80	3.41 ± 1.17
MONOA (10^9/L)	0.08 ± 0.02	0.08 ± 0.03	0.06 ± 0.02	0.06 ± 0.02
EOSA (10^9/L)	0.06 ± 0.02	0.06 ± 0.02	0.06 ± 0.02	0.07 ± 0.03
BASOA (10^9/L)	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.01	0.01 ± 0.00

Mean ± SD; n = 10 per group.

[a] = LBFLFK RBD oil was statistically significantly different ($p < 0.05$) from Kumily RBD oil.

[b] = LBFLFK RBD oil was statistically significantly different ($p < 0.05$) from both Kumily RBD oil and Menhaden oil. Menhaden oil was not compared to LBFLFK RBD oil in instances where LBFLFK RBD oil was not statistically different from Kumily RBD oil. Menhaden oil was not directly compared to Kumily RBD oil.

Table 7
Means of clinical pathology endpoints in this study compared to the historical control ranges.

Parameter	Unit	Sex	Historical control range	Kumily RBD oil	LBFLFK RBD oil (low)	LBFLFK RBD oil (high)	Menhaden oil
HGB	mmol/L	F	8.1–9.1	8.7	8.5	8.4	8.6
HCT	L/L	F	0.377–0.413	0.406	0.394	0.393	0.397
MCV	fL	F	50.6–54.6	54.3	53.3	53.0	54.3
MCH	fmol	F	1.09–1.19	1.16	1.15	1.13	1.17
TBIL	μmol/L	M	0.84–2.10	1.55	1.23	1.22	1.16
GLUC	mmol/L	F	4.36–6.40	4.91	4.93	5.43	4.96

Table 6
Rat serum chemistry values for males and females.

	Kumily RBD oil	LBFLFK RBD oil (low)	LBFLFK RBD oil (high)	Menhaden oil
Males				
ALT (μkat/L)	0.76 ± 0.18	0.72 ± 0.15	0.69 ± 0.08	0.79 ± 0.15
ALP (μkat/L)	2.43 ± 0.59	2.49 ± 0.62	2.23 ± 0.44	2.67 ± 0.73
AST (μkat/L)	2.15 ± 0.54	2.01 ± 0.35	1.78 ± 0.18	1.83 ± 0.25
GGT (nkat/L)	25 ± 0	25 ± 0	25 ± 0	25 ± 0
UREA (mmol/L)	5.18 ± 0.56	5.43 ± 0.79	5.34 ± 0.58	5.59 ± 0.48
CREA (μmol/L)	22.3 ± 1.7	21.7 ± 2.4	22.6 ± 1.9	22.9 ± 2.8
GLUC (mmol/L)	5.13 ± 0.50	5.39 ± 0.59	5.27 ± 0.42	5.20 ± 0.36
TBA (μmol/L)	37.3 ± 34.1	13.8 ± 7.3	19.8 ± 11.7	22.7 ± 13.5
TPROT (g/L)	62.64 ± 1.69	62.86 ± 1.02	62.19 ± 1.78	61.84 ± 1.24
ALB (g/L)	37.34 ± 0.87	36.96 ± 0.72	37.09 ± 0.96	37.13 ± 0.76
GLOB (g/L)	25.30 ± 1.03	25.90 ± 1.15	25.10 ± 1.01	24.72 ± 1.12
CHOL (mmol/L)	1.99 ± 0.32	1.96 ± 0.45	2.00 ± 0.24	1.32 ± 0.29
TRIG (mmol/L)	1.19 ± 0.35	1.35 ± 0.34	1.03 ± 0.21	0.93 ± 0.16
TBIL (μmol/L)	1.55 ± 0.26	1.23 ± 0.11[a]	1.22 ± 0.19[a]	1.16 ± 0.21
NA (mmol/L)	141.8 ± 0.5	141.4 ± 1.1	141.4 ± 0.7	141.3 ± 1.6
K (mmol/L)	4.73 ± 0.23	4.72 ± 0.16	4.69 ± 0.25	4.70 ± 0.23
CL (mmol/L)	96.6 ± 0.9	95.9 ± 1.2	96.3 ± 0.9	95.8 ± 1.0
INP (mmol/L)	2.15 ± 0.16	2.12 ± 0.16	2.12 ± 0.21	2.23 ± 0.18
CA (mmol/L)	2.60 ± 0.05	2.61 ± 0.06	2.59 ± 0.07	2.58 ± 0.05
Females				
ALT (μkat/L)	0.69 ± 0.09	0.65 ± 0.13	0.69 ± 0.17	0.65 ± 0.14
ALP (μkat/L)	1.16 ± 0.35	1.26 ± 0.26	1.14 ± 0.37	1.15 ± 0.28
AST (μkat/L)	1.79 ± 0.40	1.54 ± 0.25	1.62 ± 0.31	1.42 ± 0.21
GGT (nkat/L)	25 ± 0	25 ± 0	25 ± 0	25 ± 0
UREA (mmol/L)	6.23 ± 1.07	6.74 ± 0.88	6.06 ± 0.58	6.32 ± 0.66
CREA (μmol/L)	29.0 ± 3.4	28.2 ± 2.7	27.2 ± 2.4	27.7 ± 2.5
GLUC (mmol/L)	4.91 ± 0.40	4.93 ± 0.28	5.43 ± 0.43[a]	4.96 ± 0.50
TBA (μmol/L)	27.0 ± 14.9	31.5 ± 12.8	17.3 ± 8.6	28.8 ± 9.6
TPROT (g/L)	63.53 ± 2.82	63.74 ± 2.43	64.08 ± 1.72	62.78 ± 1.80
ALB (g/L)	39.48 ± 0.95	39.37 ± 1.63	39.50 ± 0.88	39.52 ± 1.14
GLOB (g/L)	24.05 ± 1.95	24.37 ± 1.41	24.59 ± 1.18	23.25 ± 1.05
CHOL (mmol/L)	1.31 ± 0.26	1.51 ± 0.48	1.33 ± 0.25	0.98 ± 0.15
TRIG (mmol/L)	0.60 ± 0.20	0.62 ± 0.25	0.48 ± 0.16	0.54 ± 0.20
TBIL (μmol/L)	1.49 ± 0.35	1.69 ± 0.31	1.44 ± 0.26	1.60 ± 0.48
NA (mmol/L)	140.6 ± 1.5	140.3 ± 1.1	140.8 ± 1.5	140.2 ± 1.8
K (mmol/L)	4.39 ± 0.27	4.47 ± 0.23	4.39 ± 0.23	4.42 ± 0.26
CL (mmol/L)	98.2 ± 1.5	97.6 ± 1.0	98.9 ± 1.3	97.7 ± 1.1
INP (mmol/L)	1.75 ± 0.18	1.80 ± 0.14	1.62 ± 0.18	1.74 ± 0.24
CA (mmol/L)	2.62 ± 0.06	2.64 ± 0.04	2.58 ± 0.06	2.58 ± 0.06

Mean ± SD; n = 10 per group.
[a] = LBFLFK RBD oil was statistically significantly different ($p < 0.05$) from Kumily RBD oil. None of these statistically significant differences were statistically significantly different from menhaden oil. Menhaden oil was not compared to LBFLFK RBD oil in instances where LBFLFK RBD oil was not statistically different from Kumily RBD oil. Menhaden oil was also not directly compared to Kumily RBD oil.

The fatty acid profile of LBFLFK RBD oil was compared with oils derived from plants, algae, fish, and a fungus, as well as edible tissues and processed products from various mammals, birds, and fish. As expected, the diversity of samples resulted in a wide range of fatty acid profiles. Each of the 48 individual fatty acids measured was detected in multiple other samples, indicating repeated dietary exposure. Principal component analysis showed that the overall fatty acid profile of LBFLFK RBD oil is most similar to the fatty acid profiles of *Mortierella alpina* oil and salmon flesh. Introduction of the EPA and DHA biosynthetic pathway in LBFLFK resulted in the production of fatty acids not normally present in conventional canola varieties. However, all fatty acids present in LBFLFK RBD oil were detected in other food products, and none of these fatty acids have been reported to cause adverse effects in humans or animals. The edible oils and fat-containing foods with the fewest differences in the presence or absence of individual fatty acids compared to LBFLFK RBD oil were the menhaden oil, fish oils, salmon oil, and *Mortierella alpina* oil, all of which are GRAS by the U.S. FDA (U.S. FDA, 1999, 2001, 2002, 2004a; b, 2017). Compared to *Mortierella alpina* oil, LBFLFK RBD oil contained three additional fatty acids (C20:3n-3, C20:3n-9, and C22:4n-3) and lacked three other fatty acids (C12:0, C16:1 trans, and C16:3n-3). The menhaden oil, fish oils, and salmon oil contained all the fatty acids present in LBFLFK RBD oil and had six additional ones that were absent from LBFLFK RBD oil, indicating a history of human consumption for all of the fatty acids

Table 8
Rat absolute organ weights for males and females.

	Kumily RBD oil	LBFLFK RBD oil (low)	LBFLFK RBD oil (high)	Menhaden oil
Males				
terminal body weight (g)	283.6 ± 17.898	289.35 ± 25.621	286.01 ± 22.27	286.77 ± 18.387
adrenal glands (mg)	62.5 ± 8.059	60.8 ± 5.996	60.8 ± 8.456	61.8 ± 5.865
brain (g)	2.066 ± 0.082	2.035 ± 0.091	2.016 ± 0.126	2.046 ± 0.101
epididymides (g)	0.781 ± 0.072	0.782 ± 0.082	0.777 ± 0.058	0.781 ± 0.047
heart (g)	0.961 ± 0.065	0.984 ± 0.090	0.974 ± 0.119	0.956 ± 0.085
kidneys (g)	2.139 ± 0.218	2.212 ± 0.249	2.168 ± 0.251	2.303 ± 0.242
liver (g)	7.356 ± 0.645	7.848 ± 0.900	7.737 ± 0.616	8.218 ± 0.693
prostate (g)	0.494 ± 0.061	0.489 ± 0.139	0.488 ± 0.071	0.562 ± 0.163
seminal vesicles with coagulating gland (g)	0.701 ± 0.129	0.666 ± 0.181	0.704 ± 0.137	0.765 ± 0.201
spleen (g)	0.589 ± 0.105	0.589 ± 0.073	0.550 ± 0.096	0.622 ± 0.094
testes (g)	3.317 ± 0.290	3.327 ± 0.311	3.437 ± 0.230	3.344 ± 0.165
thymus (mg)	484.1 ± 93.938	515.8 ± 95.087	477.8 ± 82.359	477.9 ± 96.845
thyroid glands (mg)	16.1 ± 2.079	18.1 ± 2.470	16.0 ± 3.266	17.2 ± 4.662
Females				
terminal body weight (g)	169.09 ± 13.505	176.42 ± 12.322	175.9 ± 7.722	174.13 ± 7.417
adrenal glands (mg)	64.5 ± 6.819	69.3 ± 8.354	68.4 ± 3.658	69.4 ± 11.237
brain (g)	1.861 ± 0.070	1.853 ± 0.086	1.883 ± 0.085	1.884 ± 0.055
heart (g)	0.66 ± 0.059	0.635 ± 0.043	0.637 ± 0.040	0.620 ± 0.031
kidneys (g)	1.328 ± 0.130	1.423 ± 0.120	1.372 ± 0.079	1.419 ± 0.105
liver (g)	4.398 ± 0.347	4.878 ± 0.600	4.836 ± 0.285[a]	4.947 ± 0.305
ovaries (mg)	89.4 ± 9.548	90.2 ± 10.185	94.0 ± 11.065	94.4 ± 8.656
spleen (g)	0.375 ± 0.059	0.414 ± 0.077	0.384 ± 0.048	0.388 ± 0.065
thymus (mg)	403.4 ± 57.531	460.3 ± 72.732	398.5 ± 72.124	438.2 ± 63.12
thyroid glands (mg)	15.1 ± 2.726	15.5 ± 3.866	14.9 ± 2.923	15.2 ± 2.394
uterus with cervix (g)	0.567 ± 0.230	0.578 ± 0.223	0.634 ± 0.167	0.521 ± 0.132

Mean ± SD; n = 10 per group.
[a] = LBFLFK RBD oil statistically significantly different ($p < 0.05$) from Kumily RBD oil. None of these statistically significant differences were statistically significantly different from menhaden oil. Menhaden oil was not compared to LBFLFK RBD oil in instances where LBFLFK RBD oil was not statistically different from Kumily RBD oil. Menhaden oil was also not directly compared to Kumily RBD oil.

present in LBFLFK RBD oil.

In most cases, fatty acids present in LBFLFK RBD oil were detected at lower concentrations than in other routinely consumed food products. However, six fatty acids (C18:2n-6, C18:2n-9, C18:3n-6, C20:2n-9, C20:4n-3, and C22:4n-3) were identified that had the highest relative abundance in LBFLFK RBD oil compared to the other samples included in this study. These six fatty acids are known products of the desaturases and elongases introduced into LBFLFK (Fig. 1, Yilmaz et al. (2017)).

C18:2n-6 makes up about 29% of the total fatty acids of LBFLFK RBD oil while the next closest samples, the conventional canola variety oils, have about 18–23%. C18:2n-6 is present in many plant-based oils,

Table 9
Organ/body weight ratios for males and females.

	Kumily RBD oil	LBFLFK RBD oil (low)	LBFLFK RBD oil (high)	Menhaden oil
Males				
adrenal glands	0.022 ± 0.002	0.021 ± 0.002	0.021 ± 0.003	0.022 ± 0.002
brain	0.73 ± 0.038	0.708 ± 0.072	0.707 ± 0.046	0.715 ± 0.048
epididymides	0.276 ± 0.028	0.271 ± 0.03	0.272 ± 0.021	0.273 ± 0.02
heart	0.34 ± 0.026	0.341 ± 0.024	0.34 ± 0.029	0.333 ± 0.019
kidneys	0.755 ± 0.068	0.766 ± 0.073	0.757 ± 0.49	0.082 ± 0.058
liver	2.593 ± 0.15	2.71 ± 0.158	2.706 ± 0.087	2.865 ± 0.137
prostate	0.175 ± 0.022	0.168 ± 0.043	0.171 ± 0.024	0.196 ± 0.052
seminal vesicles with coagulating gland	0.247 ± 0.041	0.229 ± 0.056	0.246 ± 0.04	0.267 ± 0.068
spleen	0.208 ± 0.037	0.205 ± 0.032	0.192 ± 0.023	0.217 ± 0.028
testes	1.172 ± 0.107	1.155 ± 0.117	1.206 ± 0.101	1.171 ± 0.101
thymus	0.17 ± 0.029	0.178 ± 0.026	0.168 ± 0.031	0.167 ± 0.037
thyroid glands	0.006 ± 0.001	0.006 ± 0.001	0.006 ± 0.001	0.006 ± 0.002
Females				
adrenal glands	0.038 ± 0.003	0.039 ± 0.004	0.039 ± 0.002	0.040 ± 0.006
brain	1.104 ± 0.058	1.054 ± 0.074	1.071 ± 0.046	1.083 ± 0.043
heart	0.391 ± 0.023	0.36 ± 0.02[a]	0.362 ± 0.020[a]	0.356 ± 0.020
kidneys	0.785 ± 0.046	0.808 ± 0.065	0.781 ± 0.044	0.815 ± 0.052
liver	2.602 ± 0.073	2.762 ± 0.241	2.751 ± 0.154	2.842 ± 0.152
ovaries	0.053 ± 0.004	0.051 ± 0.006	0.053 ± 0.005	0.054 ± 0.004
spleen	0.221 ± 0.20	0.234 ± 0.032	0.218 ± 0.024	0.223 ± 0.035
thymus	0.238 ± 0.024	0.261 ± 0.037	0.226 ± 0.036	0.252 ± 0.038
thyroid glands	0.009 ± 0.002	0.009 ± 0.002	0.008 ± 0.002	0.009 ± 0.001
uterus with cervix	0.34 ± 0.148	0.325 ± 0.106	0.36 ± 0.089	0.298 ± 0.068

Mean ± SD; n = 10 per group.
[a] = LBFLFK RBD oil statistically significantly different ($p < 0.05$) from Kumily RBD oil. None of these statistically significant differences were statistically significantly different from menhaden oil. Menhaden oil was not compared to LBFLFK RBD oil in instances where LBFLFK RBD oil was not statistically different from Kumily RBD oil. Menhaden oil was also not directly compared to Kumily RBD oil.

Table 10
Microscopic histopathology findings.

Microscopic finding	Males			Females		
	Kumily RBD oil	LBFLFK RBD oil (low)	LBFLFK RBD oil (high)	Kumily RBD oil	LBFLFK RBD oil (low)	LBFLFK RBD oil (high)
Adrenal cortex – vacuolation increased	1	0	2	0	0	0‡
Adrenal cortex – accessory cortical tissue	0	0	1	0	1	2‡
Adrenal medulla	0	0	0	0	0	0
Axillary lymph nodes – blood resorption	1	1	0	0	0	0
Axillary lymph nodes - plasmocytosis	0	0	0	0	1	0
Bone marrow	0	0	0	0	0	0
Brain	0	0	0	0	0	0
Cecum	0	0	0	0	0	0
Cervical cord	0	0	0	0	0	0
Cervix	N/A	N/A	N/A	0	0	0
Coagulating gland	0	0	0	N/A	N/A	N/A
Colon – parasite in lumen	0	0	0	0	0	1
Duodenum	0	0	0	0	0	0
Epididymides	0	0	0	N/A	N/A	N/A
Eyes with optic nerve – rosette, retinal	1	1	0	1	1	0
Eyes with optic nerve – degeneration, retinal, diffuse	0	1	0	0	0	0
Forestomach – limiting ridge: metaplasia	0	1	1	0	0	0
Forestomach – erosion/ulcer	0	0	1	0	0	0
Forestomach – inflammation, limiting ridge	0	0	0	1	0	0
Glandular stomach – dilation, glands	0	1	0	0	0	0
Heart – infiltration, lymphoid	1	0	0	0	0	0
Ileum	0	0	0	0	0	0
Jejunum	0	0	0	0	0	0
Kidneys – tubules, basophilic	8	6‡	8	6	4	5
Kidneys – mineralization, medulla	0	0‡	0	8	8	9
Kidneys – proliferation, interstitial cell	1	0‡	0	0	0	0
Kidneys - cyst	1	0‡	0	0	0	0
Kidneys – dilation, tubular	0	1‡	0	0	0	0
Liver – constriction, focal	1	0	0	0	0	1
Liver – necrosis, multifocal	1	0	0	0	0	0
Liver – infiltration, lymphoid	10	10	9	10	10	10
Liver – peri-vasculitis	1	1	0	0	0	0
Lumbar cord	0	0	0	0	0	0
Lungs – inflammation, multifocal	2	2	2	0	1	1
Lungs – crystals, eosinophilic	0	1	2	0	1	1
Lungs – hyperplasia, neuroendocrine	1	0	0	2	0	0
Lungs – osseous metaplasia	1	0	3	0	1	0
Lungs – histiocytosis, alveolar	1	1	2	1	0	1
Mesenteric lymph node – sinus histiocytosis	1	1	1	3	2	0
Ovaries	N/A	N/A	N/A	0	0	0
Peyers patch – inflammation, multifocal	1‡	0	0	0‡	0‡	0
Pituitary gland – cyst, pars distalis	0	1	2	0	1	0
Pituitary gland – cyst, pars intermedia	0	1	0	1	0	0
Pituitary gland – dilation of Ratkhe's cleft	0	2	2	3	1	2
Prostate	0	0	0	N/A	N/A	N/A
Rectum	0	0	0	0	0	0
Sciatic nerve	0	0	0	0	0	0
Seminal vesicle	0	0	0	N/A	N/A	N/A
Skeletal muscle, degeneration, hyaline	0	0	1	0	0	0
Spleen, hematopoiesis, extramedullar	0	0	1	0	1	1
Sternum with marrow	0	0	0	0	0	0
Testes – degeneration, tubular	1	1	3	N/A	N/A	N/A
Testes – hyperplasia, Leydig cell	1	0	0	N/A	N/A	N/A
Thoracic cord	0	0	0	0	0	0
Thymus - cyst	0	1	0	0	0	0
Thyroid glands	0	0	0	0	0	0
Trachea	0	0	0	0	0	0
Urinary bladder	0	0	0‡	0	0	0
Uterus - cyst	N/A	N/A	N/A	0	1	0
Vagina	N/A	N/A	N/A	0	0	0

N/A = Not applicable; n = 10 per group unless denoted by ‡ where 9 animals were examined.

with the highest levels found in grapeseed oil (58.0–78.0%), maize oil (34.0–65.6%), and safflower seed oil (67.8–83.2%) (Codex Alimentarius Commission, 2009). C18:2n-6 is also in olive oil, with percentages from 1.6 to 29.2% reported, depending on the cultivars analyzed (León et al., 2004). C18:2n-6 itself has obtained GRAS status by the U.S. FDA as a dietary supplement (U.S. FDA, 1996).

LBFLFK RBD oil contained about 1.4% of C18:2n-9, and the fish oil 1 sample had the next highest concentration, with 0.17%. C18:2n-9 is a common compound found in seafood. It is present in blue crab meat (2.63–6.51% of total fatty acids) (Çelik et al., 2004), farmed and wild gilthead (0.27 and 1.73 wt%, respectively), farmed sea bass (0.32 wt%), mussels (1.04 wt%) (Costa et al., 2017), and various fish oils (Jeong

et al., 1998; Izquierdo et al., 2005; Grigorakis et al., 2010; Beccaria et al., 2015).

LBFLFK RBD oil contained about 2.2% of C18:3n-6, and the next highest amount was in the *Mortierella alpina* oil sample, at about 1.8%. The fatty acid C18:3n-6 is present in some plant oils, such as buglossoides, borage, and evening primrose. Buglossoides oil containing 6.2–6.4% of C18:3n-6 is considered safe for use as a novel food ingredient by the EFSA Panel on Dietetic Products, Nutrition and Allergies (2015). Moreover, a refined oil from genetically engineered safflower containing about 44% C18:3n-6 is also considered GRAS by the U.S. FDA (U.S. FDA, 2016).

LBFLFK RBD oil had about 0.36% C20:2n-9. The menhaden oil and fish oil 2 samples had the next highest amounts (about 0.13%). C20:2n-9 is present in fryer rabbit muscle meat at 0.7% (Forrester-Anderson et al., 2006) and found in tissues of Gilthead bream at levels ranging from 0.1 to 0.3% of the fatty acid composition per tissue (Izquierdo et al., 2005). Trace amounts of C20:2n-9 have also been detected in melon seed oil (Hu and Ao, 2007).

LBFLFK RBD oil had about 1.9% C20:4n-3 and the menhaden oil sample had about 1.5%, which is slightly more than the reported values for other marine fish oils (less than 1%) (Ghioni et al., 2002). Additionally, C20:4n-3 is typically present in animal tissues (FAO, 2010).

C22:4n-3 was present in LBFLFK RBD oil at about 1%, and the next closest sample, menhaden oil, had about 0.1%. Evaluation of the fatty acid composition in wild sea bass showed the presence of low amounts of C22:4n-3 (0.6 ± 0.2% of total fatty acids) (Alasalvar et al., 2002). Mukhopadhyay and Ghosh (2003) reported a C22:4n-3 value of 2.9% of total lipids in carp eggs. Thus, all six of the fatty acids that had the highest relative abundance in LBFLFK RBD oil are present at similar relative abundances in other foods that were not included in this study.

In a complementary approach, a 28-day repeated dose toxicity study using Wistar rats to further assess the safety of LBFLFK RBD oil was performed. No adverse findings were identified in rats administered LBFLFK RBD oil at low or high dose. Although some isolated parameters were found to be statistically significantly different between animals given LBFLFK and Kumily RBD oils, none of these changes were statistically significantly different from values observed in the animals receiving menhaden oil and/or were within the historical control range for that endpoint. Thus, none of these observed changes were considered treatment-related or biologically relevant.

As demonstrated, all fatty acids associated with LBFLFK, when compared to commonly consumed foods from marine, animal, or plant sources, are present at similar levels and have a history of safe use and consumption. Moreover, various oils from different sources containing the fatty acids present in LBFLFK RBD oil are considered safe for consumption and use by multiple regulatory authorities such as the U.S. FDA and EFSA. The results of the 28-day repeated dose toxicity study substantiate the findings of the edible oils comparison study. When LBFLFK RBD oil was administered by oral gavage to rats at 3 mL (or 2.76 g)/kg bw/d, which corresponds at high dose to an approximately 3-fold increase in EPA and DHA concentration compared to the FDA approved daily intake of EPA and DHA based on human safety information from menhaden oil (U.S. FDA, 1999), no adverse effects were noted. In conclusion, these studies support the safety of LBFLFK RBD oil as a source of EPA and DHA for human consumption.

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Appendix A. Supplementary data

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Transparency document

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