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Effect of dietary krill oil supplementation on horse red blood cell membrane fatty acid composition and blood parameters

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Abstract

Supplementation with marine-derived *n*−3 long-chain polyunsaturated fatty acids (LC PUFAs), eicosapentaenoic acid (EPA, 20:5 *n*−3) and docosahexaenoic acid (DHA, 22:6 *n*−3) is linked to beneficial health effects in both humans and horses. Krill oil (KO), which is extracted from the Antarctic krill (*Euphausia superba*), is well documented as a safe and biologically available dietary supplement in humans and several animal species, but there is a lack of documentation regarding its effect as a dietary ingredient for horses. The objective of this study was to test whether KO as a dietary supplement had the ability to raise horse red blood cell (RBC) membrane EPA and DHA, expressed as the *n*−3 index. Five nonworking Norwegian cold-blooded trotter horse geldings (body weight [BW]: 567 ± 38 kg) were supplemented with KO (10 mL/100 kg BW) for 35 days in a longitudinal study. Blood samples were analysed for RBC membrane fatty acid (FA) profile, haematology and serum biochemistry every 7th day. KO was well accepted by all horses, and no adverse health effects were observed during the 35-day trial period. KO supplementation affected the RBC membrane FA profile by increasing the *n*−3 index from Day 0 to 35 (Day 0: 0.53% vs. Day 35: 4.05% of total RBC FAs). The observed increase in the sum of EPA and DHA ($p < 0.001$), total *n*−3 FAs ($p < 0.001$) and the reduction of *n*−6 FAs ($p < 0.044$) resulted in a lower *n*−6:*n*−3 ratio ($p < 0.001$) by Day 35 of KO supplementation. In conclusion, the RBC *n*−3 index was increased and the general *n*−6:*n*−3 ratio was decreased in horses receiving 35-day dietary KO supplementation.

KEYWORDS
DHA, docosahexaenoic acid, eicosapentaenoic acid, EPA, equine, *Euphausia superba*

1 | INTRODUCTION

Horses have the capacity to convert the essential *n*−3 polyunsaturated fatty acid (*n*−3 PUFA), alpha-linolenic acid (ALA, 18:3 *n*−3), found in various plant oils and fresh pasture grass, to the more unsaturated *n*−3 long-chain (LC) PUFA eicosapentaenoic acid (EPA, 20:5 *n*−3), while conversion of ALA to the *n*−3 LC PUFA docosahexaenoic acid (DHA, 22:6 *n*−3) has not been observed (Hansen et al., 2002; King et al., 2008). In humans, the immunomodulatory, anti-inflammatory and proresolving benefits of *n*−3 LC

PUFAs have been extensively described (Calder, 2010; Serhan et al., 2008). In horses, supplementing *n*−3 LC PUFAs has demonstrated benefits regarding various health indices, such as ameliorating equine metabolic syndrome (S. E. Elzinga et al., 2019); improving glucose clearance (O'Connor et al., 2004) and insulin sensitivity (Brennan et al., 2015; T. M. Hess et al., 2013); the general reduction of inflammatory response (Manhart et al., 2009; McCann et al., 2000); ameliorating chronic health issues such as joint health (Woodward et al., 2007), recurrent airway obstruction and inflammatory airway disease (Nogradi et al., 2015) and improved reproduction and fertility (Brinsko et al., 2005; Ferreira et al., 2021).

Several factors may influence a horse's intake of the *n*−3 and *n*−6 PUFAs so as to increase the potential benefits of dietary supplementation with *n*−3 fatty acids (FAs). Shifting from a more natural *n*−3 rich grass-based diet to a diet incorporating higher levels of *n*−6 PUFA-rich grains and plant oils may promote a dietarily less desirable, potentially more proinflammatory *n*−6:*n*−3 ratio (Simopoulos, 2002). Seasonal variations in *n*−3 content in forage plants and the conditions of forage conservation may also affect the *n*−6:*n*−3 ratio of feed (Glasser et al., 2013; Meľuchová et al., 2008). In addition, horses (Hansen et al., 2002; Vineyard et al., 2010), like humans (Arterburn et al., 2006; Burdge, 2004), show a limited capacity to convert ALA to the LC PUFAs, particularly DHA. Supplemental LC *n*−3 PUFA may be particularly beneficial for those horses with reduced access to fresh grass, performance and breeding horses and horses with health ailments such as respiratory or joint inflammation or insulin resistance (Manhart et al., 2009; Nogradi et al., 2015).

Marine fish oils are known to be rich in *n*−3 LC PUFAs and are well tolerated in horses. Krill oil (KO) is a marine dietary supplement extracted from Antarctic krill (*Euphausia superba*) (Spiridonov & Casanova, 2010). Controlled studies on KO supplementation to horses are scarce. Only one abstract has shown that KO supplementation may increase serum EPA in horses, but the level EPA and DHA in the diet was not stated (Bowen et al., 2013), hence making comparisons with other studies difficult and it can be challenging to give evidence-based feeding recommendations of KO. Antarctic krill are harvested from the wild under strict observation of the Commission for the Conservation of Antarctic Marine Living Resources (CCAMLR), an international treaty that ensures sustainable fishing with a precautionary catch quota of 1% of the biomass in one particular area off the Antarctic peninsula (Krafft et al., 2021). KO differs from most other marine oils in that it contains high concentrations of phospholipid—instead of triglyceride-bound EPA and DHA (Burri et al., 2016). This molecular form has been shown to enhance uptake into cell membranes as compared to triglyceride esters in dogs and humans (Burri et al., 2020; Ramprasath et al., 2013). A convenient measure for EPA and DHA cell membrane content, for levels at which they become biologically important, is the red blood cell (RBC) *n*−3 index. The *n*−3 index represents the EPA and DHA amounts expressed as a percentage of total RBC FAs (Harris & von Schacky, 2004). It is a validated biomarker for long-term *n*−3 PUFA intake and general *n*−3 tissue status, particularly for atrial levels in humans (Fenton et al., 2016; Harris et al., 2004; Metcalf et al., 2010).

The use of dietary supplements based on marine crustaceans in horse diets lacks documentation and to the knowledge of the authors there are no controlled studies regarding KO supplementation stating the amounts of EPA and DHA provided. The objective of the study was therefore to test whether KO can be an effective dietary supplement to increase *n*−3 LC PUFAs levels, expressed as the RBC *n*−3 index, in horses.

2 | MATERIALS AND METHODS

2.1 | Experimental design

All housing, management and experimental procedures followed the laws and regulations for experimental animals in Norway (Norwegian Government, 2015), and the experiment was approved by the Norwegian Food Safety Authority (FOTS identification number 27943). The study was designed as a longitudinal experiment over 35 days, with blood sampling on Days 0, 7, 14, 21, 28 and 35. Baseline values were measured on Day 0; then, Days 1–7 were the adaptation period to KO, and from Days 8–35, the intake of KO was kept constant. The level of KO supplementation was increased linearly from Day 0 to 7 and during the adaptation period for all horses.

2.2 | Animals and diets

Five nonworking Norwegian cold-blooded trotter horses geldings (age: 21.6 ± 4.6 years; body weight [BW]: 567 ± 38 kg [average of weekly measurements]; body condition score [Henneke et al., 1983]: 6.4 ± 0.9 [at Day 0]) were used in the study. The horses are owned by the Norwegian University of Life Sciences and are housed in a controlled management and feeding regime at the university's animal research centre. The horses were fed a daily ration (as fed) of 7.0 kg hay (first cut, Timothy grass from the same batch) and 0.3 kg sugar beet pulp (SBP; pellets soaked in water before feeding; Champion Betfiber; Felleskjøpet Fôrutvikling) top-dressed with 10 mL KO/100 kg BW (55 mL; 52.9 g) KO (QRILL™ Antarctic Phospholipid Oil; Aker BioMarine) (chemical composition of the feedstuffs is shown in Table 1). The daily dosage of 55 mL KO supplied the horses with 6.6 g EPA, 4.5 g DHA, 24.2 mg astaxanthin, 22.2 g phospholipids and 2.7 g choline. The amounts of daily dietary supplementation of EPA and DHA were calculated so as to supply a similar dosage to that which has been shown to lead to the incorporation of EPA and DHA in whole blood and plasma in previous horse studies (Nogradi et al., 2015). The horses were fed 2.0, 2.5 and 2.5 kg of hay at 0700, 1400 and 2000 h, respectively, and SBP and KO were given in two equal meals at 07:00 and 20:00 h. In addition, the horses were fed 30 g/d of sodium chloride and 100 g/d of a commercial mineral and vitamin supplement (Champion Multitiskudd; Felleskjøpet Fôrutvikling) consisting, per kg original matter, of Ca, 100 g; P, 70 g; Mg, 32 g; Cu, 840 mg; NaCl, 50 g; Zn, 2830 mg; Mn, 1530 mg; Fe, 2460 mg; I, 18 mg; Co, 6 mg; Se, 10.2 mg vitamin A, 107,000 I.U.; vitamin D, 11,300 I.U.; vitamin E, 9600 mg; vitamin B1, 260 mg; vitamin B2,

TABLE 1 Dry matter (DM, g/kg) and chemical composition (g/kg DM) of the experimental feedstuffs.

	Hay	Sugar beet pulp	Krill oil
Dry matter	905	902	1000
Ash	62	63	
Neutral detergent fibre	667	359	
Acid detergent fibre	359	205	
Acid detergent lignin	23	15	
Water soluble carbohydrates	67	96	
Crude protein	103	90	
Crude fat	19	50	963

120 mg; vitamin B6, 100 mg: vitamin B12, 0.8 mg; niacin, 270 mg; folic acid, 150 mg; biotin, 15 mg and vitamin C, 270 mg. The horses were housed in an unheated barn in 3 × 3 m individual box stalls with rubber mats and wood shavings as bedding material. They were allowed access to a dirt paddock twice a day, after meals at 0700 and 1400 h, and water was available *ad libitum* from automatic water troughs in the individual stalls, as well as buckets in the dirt paddock.

2.3 | Data collection

2.3.1 | Feedstuffs

Samples of all feedstuffs were collected once weekly, pooled and stored in sealed plastic bags at 4°C for later analysis.

2.3.2 | Blood samples

Blood samples were collected using jugular vein puncture into 10 mL heparinised tubes (BD Vacutainer, REF 367526, LH 170 I.U.; Becton, Dickinson and Company), 10 mL EDTA tubes (Vacuette, REF 455036, K3E K3EDTA; Greiner Bio-One GmbH) and 10 mL serum tubes (BD Vacutainer, CAT, REF 367896; Becton) at 1200 h on every sampling day. The samples in heparinised tubes were centrifuged immediately after sampling at 3000*g* for 10 min (Heraeus labofuge 300; Thermo Fisher Scientific), and plasma and RBC were harvested separately and stored at −80°C for later analysis. Samples of biochemical and haematological analysis were delivered within 30 min after sampling and analysed within 5 h.

2.4 | Analysis of feed and blood samples

2.4.1 | Feed samples

Feed samples of hay and SBP were milled so as to pass through a 1 mm metal screen (cutting mill SM 200; Retsch GmbH) and analysed in duplicate for dry matter (DM), ash, crude protein (CP), neutral detergent fibre (NDF) and acid detergent fibre (ADF), water-soluble carbohydrates (WSC), crude fat (CF) and FA composition. The KO was analysed to determine its CF and FA composition. DM content was measured by drying to a constant weight (24 h at 105 ± 2°C), and samples were incinerated at 550°C for 16 h for ash determination. The WSC were determined as described previously (Randby et al., 2010). Nitrogen was determined according to the Kjeldahl method (AOAC International, 2001), and CP was calculated as N × 6.25. CF was analysed according to the accelerated solvent extractor method (Dionex ASE 350; Thermo Fisher Scientific). NDF was assayed with a heat-stable amylase and expressed including residual ash (aNDF), ADF was expressed including residual ash and ADL for all samples were measured by the filter bag technique described by ANKOM (2017a, 2017b).

2.4.2 | Feed FA analysis

The FA compositions of the feed samples were determined via gas chromatography (GC) using a Trace 1300 GC with an AS1310 auto-sampler and a flame ionization detector (Thermo Fisher Scientific). Lipid extraction and direct methylation were performed in accordance with O'Fallon et al. (2007). The fatty acid methyl esters were subsequently separated using an RTSP-2560 capillary column (100 × 0.25 mm internal diameter [ID] × 0.20 µm film thickness, from RESTEK). The carrier gas was helium, and the pressure was 270 kPa. The initial oven temperature was 140°C. This was held for 5 min, subsequently increased to 240°C at a rate of 4°C per min, and then held for 20 min. The FA analysis was performed via the auto-injection of 1 µL of each sample at a split ratio of 40:1, a helium flow of 1 mL/min and a temperature of 260°C. The flame ionisation detector temperature was 260°C, with helium, air, and N_2 exhibiting gas flow rates of 1, 300 and 27 mL/min respectively. The sampling frequency was 10 Hz. The run time for a single sample was 50 min. The identification of FA peaks, as determined by GC, was then used to calculate the amounts of FAs (g/100 g fat) based on theoretical response factors (Ackman & Sipos, 1964).

2.4.3 | RBC membrane FA analysis

The RBC membrane FA composition and the *n*−3 index were analysed by OmegaQuant Analytics. OmegaQuant is a Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory, and the assay for FA analysis has been validated and standardised (Harris et al., 2013). GC was carried out using a GC-2010 (Shimadzu Corporation) equipped with an SP-2560 100-m fused silica capillary column (0.25 mm ID, 0.2 µm film thickness; Supelco). The FAs were identified and calibrated using a standard mixture of defined FAs (GLC OQ-A; NuCheck Prep). The composition of FAs was expressed

as a percentage of the total identified FAs, and the *n*−3 index is defined as the sum of the percent fractions of EPA and DHA (Harris & von Schacky, 2004).

2.4.4 | Haematology and serum biochemistry

All haematological and serum biochemistry analyses were performed at the Central Laboratory at the Norwegian University of Life Sciences. The haematological parameters, including a five-part differential count, were assayed with an ADVIA® 2120 Hematology System using ADVIA 2120 MultiSpecies™ System Software (Siemens Healthcare Diagnostics). Blood smears were evaluated by an experienced technician for the visual validation of the instrument measurements and assessments of blood cell morphology.

All serum samples were evaluated for hemolysis and lipemia. Five enzymes (aspartate aminotransferase [AST], alkaline phosphatase [ALP], creatine kinase [CK], gamma-glutamyl transferase [GGT], glutamate dehydrogenase [GD]), six metabolites (total protein, albumin, urea, creatinine, total bilirubin, glucose), six minerals (Ca, P, Mg, Na, K, Cl) and SAA (serum amyloid A) were assayed with an Atellica® CH 930 Analyzer (Siemens Healthcare Diagnostics). Globulins were calculated as the difference between total protein and albumin. The analytical methods were routinely calibrated according to the manufacturer's recommendations. Control samples, both commercially available samples and internal control samples, were analyzed before each sample run.

2.5 | Statistical analysis

Blood samples and BW were analysed via repeated measurements using the MIXED procedure in SAS® (Version 9.4; SAS Institute), where the model was comprised of the effect of time (Day 0, 7, 14, 21, 28 and 35) and the random effect of the horse. Serial correlation over time was modelled using a spatial Gaussian correlation structure. Data were assessed for normality by Shapiro–Wilk test in GraphPad Prism (version 8.0.2; GraphPad Software). Some of the haematological and serum biochemistry data were log transformed, but it did not affect the analysis. Results are presented as means, and standard errors of the means (SEMs) are reported. Effects would be considered significant if $p < 0.05$.

3 | RESULTS

3.1 | Feeds and feeding

All horses consumed the allocated hay, SBP and KO, and no leftovers were observed. The horses accepted the KO supplement well, and no adverse effects, such as an allergic reaction or gastrointestinal issues, were detected by those involved in animal care or the in-house veterinarian. FA compositions of the feedstuffs are presented in Table 2. KO was the only feedstuff in the daily feed ration containing EPA (20:5 *n*−3) and DHA (22:6 *n*−3).

TABLE 2 Fatty acid (FA) composition (g/100 g FA) of the experimental feedstuffs.

Fatty acid	Hay	Sugar beet pulp	Krill oil
C10:0	0.00	0.00	0.00
C12:0	0.27	0.00	0.10
C14:0	0.52	0.09	10.99
C14:1n7	0.11	0.00	0.17
C15:0	1.30	0.12	0.45
C15:1	0.00	0.00	0.00
C16:0	20.48	15.18	23.30
C16:1n7	1.00	0.00	4.20
C17:0	0.23	0.00	0.36
C17:1	0.00	0.00	0.00
C18:0	1.56	3.57	1.45
C18:1n9t	0.00	0.00	0.18
18:1n9c	2.96	20.27	10.88
C19:0	0.00	0.00	0.00
C18:2n6t	0.00	0.00	0.00
C18:2n6c	22.45	52.37	1.67
C20:0	1.00	0.36	0.10
C18:3n6	0.00	0.00	0.23
C20:1	0.00	0.00	0.53
C18:3n3	46.22	7.24	3.38
C21:0	0.00	0.00	0.00
C20:2	0.00	0.00	7.83
C22:0	1.20	0.42	0.02
C20:3n6	0.00	0.00	0.13
C22:1n11	0.00	0.00	0.24
C22:1n9	0.00	0.00	0.47
C20:3n3	0.00	0.00	0.27
C23:0	0.00	0.00	0.00
C20:4n6	0.00	0.12	0.41
C22:2	0.00	0.00	0.22
C24:0	0.71	0.27	0.00
C20:5n3	0.00	0.00	19.31
C24:1	0.00	0.00	0.00
C22:5n3	0.00	0.00	0.47
C22:6n3	0.00	0.00	12.54

The BW of the horses did not change during the experiment and was on average 567 ± 38 kg.

3.2 | FA parameters

The effect of KO supplementation on RBC membrane FAs is presented in Table 3. There was an effect on the part of time ($p < 0.05$) on all FAs, except palmitoleic, oleic, behenic, dihomo-γ-linolenic and docosatetraenoic acids. The levels of EPA and DHA, and the sum of these two FAs increased from Day 0 to 35 ($p < 0.001$), increasing the *n*−3 index (Day 0: 0.53 vs. Day 35: 4.05% of total RBC FAs). Although the level of *n*−6 arachidonic acid (AA) showed a slight increase (1.19% to 1.40% of RBC membrane FAs) from Day 0 to 35 ($p < 0.001$), the proportionally higher increase in EPA (0.422% to 2.92% of RBC membrane FAs) led to an overall decrease in the AA:EPA ratio (Figure 1). From Day 0 to 35, the *n*−3 FAs increased ($p < 0.001$), and the *n*−6 FAs decreased ($p < 0.044$), resulting in a lower ($p < 0.001$) *n*−6:*n*−3 ratio at the end of the study (Figure 1).

3.3 | Biochemical parameters

The effect of KO supplementation on plasma biochemical parameters is presented in Table 4. There was an effect of time on the three serum enzymes AST ($p = 0.01$), ALP ($p < 0.001$) and GGT ($p < 0.001$), while no effect was seen for CK or GD. For AST, there was a reduction from Day 0 to 7 ($p = 0.01$), which remained until Day 35, while for ALP, there was a continuous weekly reduction in measurements from Day 0 through Day 35 ($p < 0.001$). The GGT values fluctuated, being reduced on Days 7, 14 and 28, while increasing again on Days 14 and 35, with an overall decrease from Days 0 to 35 ($p < 0.001$). For SAA, an acute

TABLE 3 Red blood cell membrane fatty acids (FA) expressed as a percentage of the total identified FA.

		Day 0*	Day 7**	Day 14	Day 21	Day 28	Day 35	SEM	*p* Value
Myristic acid	C14:0	0.24^{c}	0.25^{bc}	0.27^{a}	0.27^{a}	0.27^{ab}	0.27^{ab}	0.015	0.002
Palmitic acid	C16:0	15.1^{a}	15.0^{a}	15.1^{a}	14.7^{b}	14.7^{b}	14.7^{b}	0.48	0.004
Palmitelaidic acid	C16:1n7t	0.064^{c}	0.079^{b}	0.087^{b}	0.090^{ab}	0.088^{b}	0.102^{a}	0.010	<0.001
Palmitoleic acid	C16:1n7	1.68	1.66	1.58	1.59	1.65	1.59	0.041	0.117
Stearic acid	C18:0	16.8^{b}	16.7^{b}	17.5^{a}	16.3^{b}	16.2^{b}	16.3^{b}	0.34	0.003
Elaidic acid	C18:1t	0.176^{c}	0.172^{c}	0.201^{b}	0.197^{b}	0.200^{b}	0.218^{a}	0.0094	<0.001
Oleic acid	C18:1n9	23.5	23.0	21.5	22.4	22.9	22.4	0.51	0.117
Linoelaidic acid	C18:2n6t	0.175^{ab}	0.159^{b}	0.161^{b}	0.183^{b}	0.172^{ab}	0.178^{a}	0.010	0.032
Linoleic acid	C18:2n6	32.3^{a}	32.1^{a}	32.0^{a}	31.8^{ab}	30.9^{bc}	30.8^{c}	0.36	0.015
Arachidic acid	C20:0	0.484^{b}	0.496^{b}	0.489^{b}	0.505^{b}	0.535^{a}	0.535^{a}	0.018	<0.001
Gamma-linolenic acid	C18:3n6	0.219^{a}	0.220^{a}	0.204^{b}	0.199^{b}	0.213^{a}	0.195^{b}	0.005	<0.001
Eicosenoic acid	C20:1n9	0.469^{a}	0.465^{a}	0.438^{b}	0.425^{b}	0.437^{b}	0.421^{b}	0.015	<0.001
Alpha-linolenic acid	C18:3n3	3.54^{a}	3.31^{b}	3.05^{c}	3.00^{c}	2.99^{c}	2.81^{d}	0.09	<0.001
Eicosadienoic acid	C20:2n6	0.240^{a}	0.237^{a}	0.233^{ab}	0.216^{c}	0.219^{bc}	0.214^{c}	0.017	0.003
Behenic acid	C22:0	0.590	0.602	0.563	0.604	0.631	0.622	0.032	0.212
Dihomo-g-linolenic acid	C20:3n6	0.297	0.305	0.326	0.312	0.307	0.327	0.016	0.129
Arachidonic acid	C20:4n6	1.19^{b}	1.25^{b}	1.37^{a}	1.31^{a}	1.29^{ab}	1.40^{a}	0.10	<0.001
Lignoceric acid	C24:0	1.50^{bc}	1.61^{ab}	1.36^{c}	1.69^{a}	1.73^{a}	1.70^{a}	0.11	0.008
Eicosapentaenoic acid	C20:5n3	0.422^{e}	1.12^{d}	1.91^{c}	2.28^{b}	2.53^{b}	2.92^{a}	0.17	<0.001
Nervonic acid	C24:1n9	0.380^{b}	0.468^{b}	0.366^{b}	0.543^{a}	0.517^{a}	0.520^{a}	0.040	0.002
Docosatetraenoic acid	C22:4n6	0.062	0.060	0.058	0.053	0.059	0.062	0.006	0.766
Docosapentaenoic—n6 acid	C22:5n6	0.049^{c}	0.057^{bc}	0.058^{bc}	0.066^{ab}	0.070^{a}	0.070^{a}	0.008	0.006
Docosapentaenoic—n3 acid	C22:5n3	0.36^{c}	0.38^{c}	0.50^{b}	0.48^{b}	0.53^{b}	0.60^{a}	0.04	<0.001
Docosahexaenoic acid	C22:6n3	0.11^{e}	0.30^{d}	0.65^{c}	0.81^{b}	0.92^{b}	1.14^{a}	0.09	<0.001

Note: Values within a row are different if superscripts ($^{a, b, c, d, e}$) differ ($p < 0.05$).

*Day 0: Start of 7-day adaptation period.

**Day 7: Last day of adaptation period.

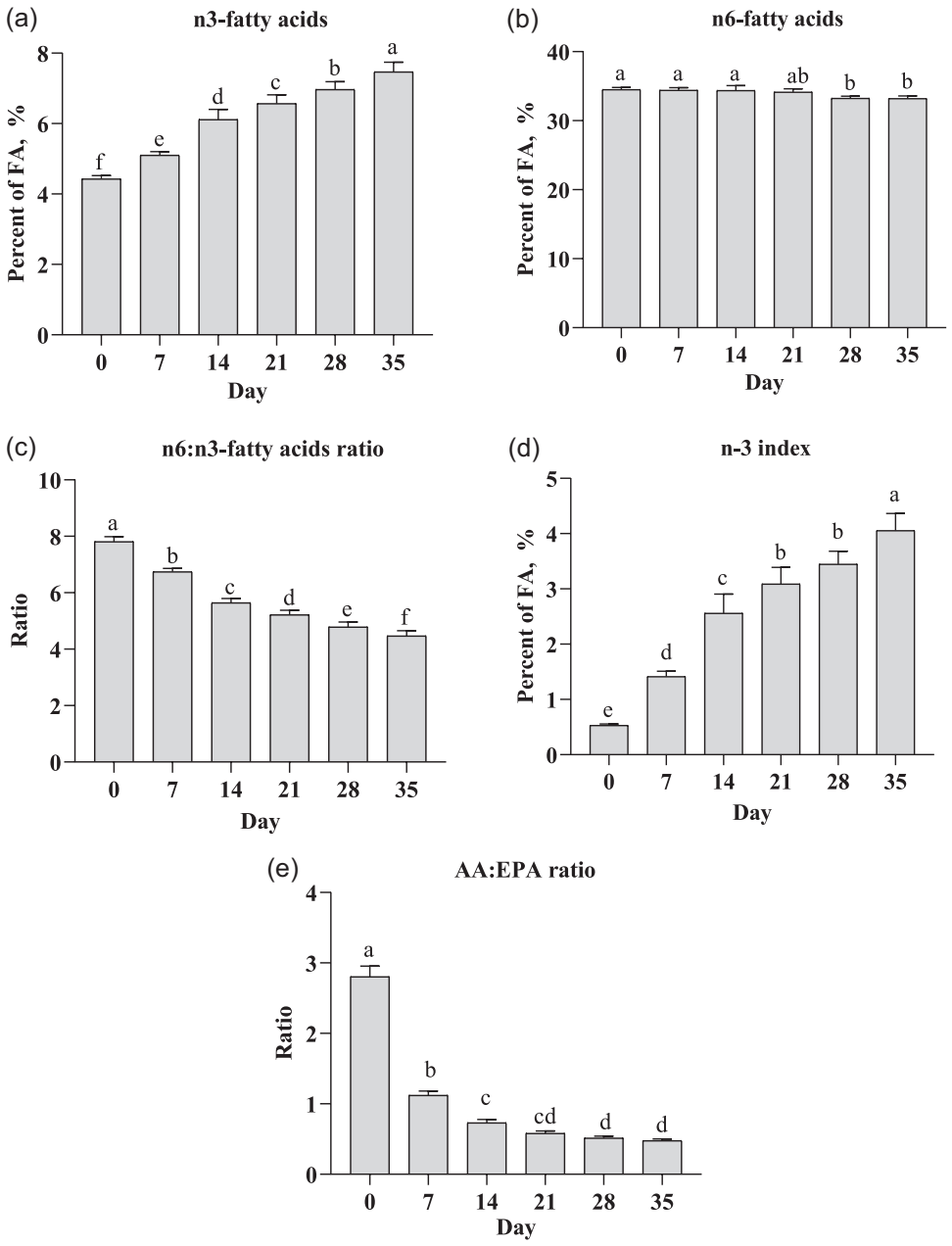

FIGURE 1 Red blood cell (RBC) membrane fatty acids (FA) from Day 0 to 35 of the study. RBC membrane (a) *n*−3, (b) *n*−6 and (d) EPA + DHA FAs expressed as a percentage of total identified FAs and (c) the ratios of n6:n3 and (e) AA:EPA FAs. Values with different superscripts differ ($p < 0.05$).

phase protein, no change was measured for the first 3 weeks (Days 0, 7 and 14), but an increase ($p < 0.001$) was measured for the last 2 weeks of the study (Days 28 and 35). The reference range for SAA is between 0 and 20 mg/L, and the horses showed an average increase from 0 to 1.2 mg/L.

3.4 | Metabolites

For the metabolites total protein, globulins, urea, creatinine and glucose, there were mild fluctuations ($p < 0.001$) in the measured values from Day 0 to 35 (Table 4). For albumin, albumin/globulin ratio and total bilirubin, there were no changes in the measured values from Day 0 to 35 of the study (Table 4).

3.5 | Minerals

All mineral measurements were within the normal reference range for cold-blooded horses for the duration of the study (Table 4). Variations seen between weekly measurements of Ca, P, Mg, Na and K levels between Day 0 and 35 were not considered to be due to

TABLE 4 Haematology and serum biochemistry in blood samples collected from Day 0 to 35 of the study.

	Units	Reference	Day 0*	Day 7**	Day 14	Day 21	Day 28	Day 35	SEM	*p* Value
Biochemical parameters										
Aspartate aminotransferase	U/L	0–500	308^{a}	274^{b}	270^{b}	266^{b}	272^{b}	265^{b}	18	0.010
Alkaline phosphatase	U/L	0–350	142^{a}	126^{b}	112^{c}	103^{cd}	97de	92^{e}	18	<0.001
Creatine kinase	U/L	0–600	175	184	153	148	158	155	16	0.376
Gamma-glutamyl transferase	U/L	0–50	21.8^{a}	12.4^{c}	20.0^{ab}	13.0^{c}	10.2^{c}	18.8^{b}	2.0	<0.001
Glutamate dehydrogenase	U/L	0–15	2.40	1.40	2.00	2.40	2.60	1.80	0.63	0.606
Serum amyloid A	Mg/L	0–20	0.00^{c}	0.00^{c}	0.00^{c}	0.20^{bc}	0.60^{b}	1.20^{a}	0.15	<0.001
Metabolites										
Total protein	g/L	58–75	67.2^{ab}	63.6^{cd}	68.0^{a}	67.0^{ab}	65.4^{bc}	61.8^{d}	1.7	<0.001
Albumin	g/L	26–36	31.8	31.0	32.2	32.0	31.4	31.8	0.8	0.292
Globulins	g/L	25–44	35.4^{a}	32.6^{b}	35.8^{a}	35.0^{a}	34.0^{ab}	30.0^{c}	1.4	<0.001
Albumin:globulin ratio		0.6–1.4	0.90^{b}	0.92^{b}	0.88^{b}	0.92^{b}	0.92^{b}	1.04^{a}	0.04	<0.001
Urea	mmol/L	3.1–8.7	3.84^{b}	4.74^{a}	4.42^{a}	4.36^{a}	4.62^{a}	4.92^{a}	0.27	<0.001
Creatinine	μmol/L	86–170	117^{c}	119^{c}	125^{b}	125^{b}	133^{a}	133^{a}	4.4	<0.001
Bilirubin	μmol/L	08–60	18.4	20.6	19.2	20.4	23.2	22.6	3.4	0.066
Glucose	mmol/L	4.0–9.0	4.90^{ab}	4.80^{ab}	4.60^{bc}	4.88^{ab}	4.34^{c}	5.06^{a}	0.19	0.005
Minerals										
Phosphorus	mmol/L	0.8–1.8	1.30^{a}	1.06^{c}	1.20^{b}	1.18^{b}	1.14^{bc}	1.06^{c}	0.05	<0.001
Calcium	mmol/L	2.7–3.2	3.02	2.98	3.02	2.96	2.92	3.02	0.05	0.182
Magnesium	mmol/L	0.7–1.2	0.72^{c}	0.76^{bc}	0.74^{bc}	0.78^{ab}	0.76^{bc}	0.82^{a}	0.03	0.023
Sodium	mmol/L	134–144	139^{a}	136^{c}	139^{a}	140^{a}	140^{a}	138^{b}	0.69	<0.001
Potassium	mmol/L	3.0–5.2	3.52^{b}	3.74^{b}	3.86^{ab}	3.78^{b}	3.40^{b}	4.46^{a}	0.24	0.048
Chloride	mmol/L	96–106	103^{a}	103^{a}	104^{a}	104^{a}	104^{a}	102^{b}	0.67	<0.001
Haematology										
White blood cells	$\times10^{9}$/L	5.0–12.0	6.82	6.56	6.66	6.26	6.46	5.86	0.811	0.084
Red blood cells (RBC)	$\times10^{12}$/L	6.0–11.0	7.26	7.06	7.42	7.24	7.64	7.06	0.34	0.138
Haemoglobin	g/L	100–175	127	120	126	124	130	120	3.5	0.093
Haematocrit	L/L	0.28–0.50	0.378	0.372	0.388	0.376	0.388	0.354	0.011	0.093
Mean erythrocyte volume	fL	36–52	51.8^{b}	52.4^{a}	52.6^{a}	52.2^{ab}	50.8^{c}	50.8^{c}	1.3	<0.001
Mean corpuscular haemoglobin concentration	g/L	335–385	336^{b}	327^{c}	325^{c}	331^{b}	337^{ab}	340^{a}	1.9	<0.001
RBC distribution width	%	16–22	20.0^{ab}	19.6^{b}	19.4^{b}	20.0^{a}	20.2^{a}	19.8^{ab}	0.4	0.015
Thrombocytes	$\times10^{9}$/L	90–350	159^{a}	132^{bc}	132^{bc}	129^{c}	138^{b}	136^{bc}	10	<0.001
Neutrophil granulocytes	$\times10^{9}$/L	2.1–7.0	4.24^{a}	4.08^{a}	3.92^{ab}	3.54^{bc}	3.58^{bc}	3.30^{c}	0.77	0.008
Lymphocytes	$\times10^{9}$/L	1.5–5.0	2.10^{c}	2.04^{c}	2.28^{bc}	2.30^{b}	2.44^{ab}	2.16^{c}	0.20	0.028
Monocytes	$\times10^{9}$/L	<1.0	0.30^{a}	0.24^{bc}	0.28^{ab}	0.28^{ab}	0.26^{ab}	0.22^{c}	0.04	0.040
Eosinophile granulocytes	$\times10^{9}$/L	<0.8	0.14	0.14	0.12	0.12	0.14	0.06	0.02	0.159
Basophile granulocytes	$\times10^{9}$/L	<0.3	0	0	0	0	0	0		
Large unstained cells	$\times10^{9}$/L	<0.3	0	0	0	0	0	0		

Note: Values within a row are different if superscripts ($^{a, b, c, d, e}$) differ ($p < 0.05$).

*Day 0: Start of 7-day adaptation period.

**Day 7: Last day of adaptation period.

the consumption of the supplemental KO. For Ca, there were no measured differences between Days 0 and 35.

3.6 | Haematology

The measured values for white blood cells (WBC), RBC, haemoglobin and haematocrit remained unchanged from Day 0 to 35, and the slight but significant changes to mean erythrocyte volume (MCV), mean corpuscular haemoglobin concentration (MCHC), RBC distribution width (RDW), thrombocytes (PLT) and leukocytes were all within normal reference ranges for healthy horses and were not considered to be due to the consumption of the supplemental KO (Table 4).

4 | DISCUSSION

In humans (Bilinski et al., 2020), dogs (Burri et al., 2020) and other animals (Burri & Johnsen, 2015), KO has been shown to be a viable option as an *n*−3 LC PUFA dietary supplement. The primary objective of the current study was to evaluate the effect of a 35-day supplementation of KO on the *n*−3 index and other RBC membrane FAs in horses. To the knowledge of the authors, this is the first controlled study demonstrating that supplementing horses with a known daily dose of 6.6 g EPA and 4.5 g DHA has the anticipated effects on these blood variables. All horses readily consumed the KO as a top dressing and remained healthy throughout the study. No adverse effects, such as allergic reactions or gastrointestinal issues, were detected by those involved in animal care. These observations are in agreement with studies that involved supplementing horses with other marine-based FA supplements (Pearson et al., 2022). In agreement with earlier studies of KO supplementation in both humans (Bilinski et al., 2020) and dogs (Burri et al., 2020), the daily supplementation with 55 mL KO (supplying 6.6 g EPA and 4.5 g DHA) performed in the current study significantly raised the *n*−3 index of the horses. Other dietary *n*−3 PUFA sources, such as fish oils, have also been shown to influence the *n*−3 index in horses (T. M. Hess et al., 2012; Vineyard et al., 2010).

An increase in the *n*−3 index was already seen after the first adaptation week. This observation is in agreement with earlier studies on *n*−3 LC PUFA supplementation in horses. King et al. (2008) measured the greatest plasma concentration change in EPA and DHA by Day 3 of a 28-day supplementation period. The same study observed that a dose-related peak level was reached on Day 7 of *n*−3 FA supplementation. In the current study, a gradual weekly increase was observed in RBC, EPA and DHA levels from pre-supplementation to Day 35 of supplementation. T. M. Hess et al. (2012) observed similar changes to RBC membrane FA compositions when supplementing with a marine *n*−3 oil in horses. They reported an increase in EPA from Day 0 to 30 and from Day 30 to 60 but no further increase from Day 60 until Day 90 of supplementation. For DHA, they observed a gradual increase in RBC levels from Day 0 to 90 (T. M. Hess et al., 2012). Other studies have observed plasma DHA reaching a peak on Day 40 of supplementation (Nogradi et al., 2015). Prior to the onset of the current study, the horses were fed a pure forage and grass diet devoid of AA. The observed slight increase in RBS membrane AA level may therefore be seen in relation to the natural level of *n*−6 AA in KO (Table 2), and similarly, slight increases in AA have been reported for other studies in horses receiving marine oil supplementation (Vineyard et al., 2010).

In addition to EPA, DHA and AA, there was a significant increase in myristic (C14:0), palmitelaidic (C16:1n7t), elaidic (C18:1t), arachidic (C20:0) lignoceric (C24:0), nervonic (C24:1n9), docosapentaenoic (C22:5n6) and docosapentaenoic (C22:5n3) FAs. The increase seen for the above-mentioned FAs may only be achievable with a compensatory reduction in other RBC membrane FAs. In the current study, there was a reduction in the levels of palmitic, LA, gamma-linolenic, eicosenoic and eicosadienoic acids from the second to the third weeks of KO supplementation, as well as a reduction in ALA after the first acclimatising week. The overall increase in *n*−3 PUFA and reduction in *n*−6 PUFA resulted in an overall reduction in the *n*−6:*n*−3 PUFA ratio (Figure 1). This may be of relevance for the health of horses because an increase in *n*−3 FAs is known to have anti-inflammatory properties, as compared to the more proinflammatory properties of *n*−6 PUFAs (T. Hess & Ross-Jones, 2014). These results are in accordance with studies conducted with other animals, such as dogs (Dominguez et al., 2021), and confirm studies showing that the increase of *n*−3 PUFAs and the *n*−3 index is proportional to the level of *n*−3 LC PUFAs supplemented into the diet (King et al., 2008; Pearson et al., 2022). The results of the present study show that supplementing horses for 28 days with the given dose of KO and *n*−3 LC PUFA level may lead to an increased *n*−3 index, potentially improving inflammatory-related health issues in horses (Nogradi et al., 2015; Woodward et al., 2007).

To the authors knowledge, there are currently no studies that specify the lower level of *n*−3 LC PUFA supplementation needed to achieve health beneficiary effects in horses. Pearson et al. (2022) reported a dose-dependent rise in horse whole blood *n*−3 LC PUFA following 6 weeks of dietary supplementing two groups of horses fed 1.8 g EPA and 3.6 g DHA per day, indicating that a lower dose of KO supplementation may have been sufficient to result in measurable changes in plasma *n*−3 LC PUFA. The daily dose of 6.6 g EPA and 4.5 g DHA fed to the horses in the current study were comparable to the lower dose fed by King et al. (2008) and supplied only a slightly higher level of DHA compared to that indicating beneficial effect for horses with chronic lower airway inflammatory disease (Nogradi et al., 2015). Woodward et al. (2007) found little effect on trot stride length and lameness score in horses treated with 4.6 g EPA + DHA per day, while Manhart et al. (2009) and Ross-Jones et al. (2014) reported that supplementing 34.8 g and 34.2 g EPA + DHA per day respectively, may be advantageous for horses with arthritis and results tended to lower synovial fluid PGE_2. Bazzano et al. (2015) reported that a 30-day supplementation of 19.2 g EPA + DHA per day might have beneficial effects on performance when using an erythrocyte osmotic fragility.

In humans, non-dietary factors such as sex and age may influence blood tissue levels of *n*−3 LC PUFA (de Groot et al., 2019) and sex-related differences in metabolic fate of *n*−3 LC PUFA in animals supplemented with marine *n*−3 sources are reported (Ghasemifard et al., 2015). However, earlier studies in horses evaluating the ability of marine *n*−3 dietary supplements using mares, geldings and/or stallions have not reported considerations regarding potential effects of sex on the metabolism of *n*−3 LC PUFA (Brinsko et al., 2005; T. M. Hess et al., 2012; Munsterman et al., 2005; Nogradi et al., 2015; O'Connor et al., 2007). Furthermore, S. Elzinga et al. (2014) found no differences in total tract macronutrient digestibility between healthy adult and aged horses, indicating that results of the current study are relevant for horses of all ages and gender.

An analysis of general blood parameters is useful for evaluating general health status and widely used in diagnosis, monitoring response to treatments and evaluating an animal's metabolic state (Messer, 1995). All the biochemical and haematological parameters measured in the current study were within the reference range specified for horses (NMBU, 2022), confirming that the horses were in good health before and during the KO-supplementation period. The results of the current study are in accordance with both human and animal studies, enabling the conclusion that KO may be considered a safe and well-tolerated *n*−3 LC PUFA source for horses (Burri et al., 2020; Robertson et al., 2014; Ulven et al., 2011).

One limitation to this study is the lack of a control group. Despite the lack of a control group, the clear increase in the *n*−3 index from Day 0 to 35 of the trial period may be seen as an effect of the dietary KO supplementation, as prior to Day 0 none of the horses had been fed KO or any other form of *n*−3 dietary supplement. Another limitation is the length of the study, as the *n*−3 index may still have increased after 35 days of supplementation. It is not known what level the *n*−3 index would have reached if the horses were supplemented for a longer time. In a comparable study a plateau was reach within 28 days of supplementation with fish oil, another EPA and DHA rich feedstuff (King et al., 2008). The comparison of KO with fish oil would be of interest in future studies.

5 | CONCLUSIONS

The study is the first controlled study demonstrating that supplementing horses with a known daily dose of 6.6 g EPA and 4.5 g DHA from KO alters the RBC membrane FA profile. This was demonstrated by a gradual increase in the *n*−3 index and decrease in the *n*−6:*n*−3 ratio during 35 days of supplementation.

AUTHOR CONTRIBUTIONS

Study conception and design were carried out by Nicole Frost Nyquist and Rasmus Bovbjerg Jensen. Manuscript preparation was carried out by Nicole Frost Nyquist, Lena Burri and Rasmus Bovbjerg Jensen. Rasmus Bovbjerg Jensen performed the statistical analysis. All authors have read and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors Nicole Frost Nyquist and Rasmus Bovbjerg Jensen declare that they have no competing interests. Lena Burri is working for Aker BioMarine Antarctic AS.

DATA AVAILABILITY STATEMENT

The datasets used during the current study are available from the corresponding author upon reasonable request.

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