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Effect of dietary linseed oil supplementation on nutrients digestibility, *in vitro* gas production and blood parameters in horses

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ABSTRACT. High-starch diets may cause metabolic disorders and hyperactivity in sport horses. Concentrates based on cereal grains, rich in fermentable starch, can be partially replaced with vegetable oils. Linseed oil provides essential fatty acids and has a favourable omega-6 to omega-3 ratio. However, limited data exist on its dose-dependent effects on digestion and physiology in horses. This study assessed the impact of two doses of dietary linseed oil on nutrient digestibility and selected blood parameters in non-working horses. Additionally, an *in vitro* gas production (GP) test evaluated microbial fermentation of selected feeds. Six Konik Polski mares (3–12 years, 200–370 kg body weight) were fed meadow hay and a concentrate supplemented with 0, 150, or 300 ml/day of linseed oil. The study was an *in vivo* digestion experiment employing a 3 × 3 Latin square design. Digestibility was determined using the acid-insoluble ash (AIA) marker method. Faecal inoculum was used for GP testing with oat grain and meadow hay. Blood samples were collected before and after feeding to analyse haematological and biochemical parameters. Linseed oil significantly improved ether extract digestibility but had no effect on other nutrients. *In vitro* GP tended to decrease in high-fibre feed with oil supplementation. The 150 ml dose lowered haemoglobin and packed cell volume, while 300 ml increased these values. Significant changes were observed in total protein, albumin, urea, bilirubin, and cholesterol concentrations. Linseed oil supplementation did not adversely affect nutrient digestibility and showed dose-dependent influences on blood parameters, indicating its potential as an alternative energy source in horse nutrition.

Introduction

A high proportion of starch in the diet may lead to several metabolic disorders and cause hyperactivity in sport horses (Warren and Vineyard, 2013). While high starch inclusion can be avoided in leisure horses it is more likely to feature in the diet of sports horses. Feeding starch should be limited to <2 g starch/kg/BW/meal to avoid rapid fermentation in the hindgut (Julliand et al., 2006). Concentrates commonly used in horse feeding contain predominantly different types of cereal grains as the main energy sources, which are rich in fast-released energy. Replacing part of the grain with vegetable oil enables an increase in the energy density of the diet and helps to maintain the good health of the animal (Warren and Vineyard, 2013). Moreover, the high concentration of energy in oils allows for a decreased volume of feed consumed, which is a critically important in the diet of race and

endurance horses, as it is inadvisable to overload the digestive tract with an excessive amount of feed (Kronfeld and Harris, 2003).

The use of high amounts of complementary feed mixture in performance horses leads to an undesirable increase in the omega-6 to omega-3 fatty acid (FA) ratio. In a hay-based diet, the ratio between omega-6 and omega-3 FA equals 0.6:1 (Warren and Kivipelto, 2007). In the pasture, this ratio equals 0.3:1, whereas in a diet consisting of concentrate and hay, it ranges from 2:1 to 7.8:1, depending on the amount and quality of fat used for the production of the mixture. This negative disproportion can be reduced by using linseed oil, which is rich in omega-3 acids.

Besides energy, oils provide biologically active compounds like essential fatty acids (EFA). In this regard, vegetable oils are characterized by a high portion of polyunsaturated fatty acids (PUFAs), such as linoleic acid (LA; 18:2n-6) and α-linolenic acid (ALA; 18:3n-3), whose consumption is associated with multiple health benefits for animals. PUFAs take part in the transportation and oxidation of cholesterol and also build cellular membranes by synthesizing crucial chemical compounds (Frota et al., 2010). Omega-3 and omega-6 FA are metabolised by the same enzymes, which means they compete with each other. Omega-3 FA metabolites have anti-inflammatory properties and contribute to the inhibition of platelet aggregation (PLT). Metabolites of omega-6 FA have an antagonistic effect, potentially exerting pro-inflammatory and prothrombotic effects in the body (Materac et al., 2013). Mammals are unable to synthesize them internally due to a lack of specific enzymes; therefore, PUFAs need to be delivered with the diet (Warren and Vineyard, 2013). The American requirement standard for horses (NRC, 2007) recommends LA at a 0.5% of daily dry matter (DM) intake.

The administration of supplements containing only isolated LA or ALA in the form of tablets or capsules generates numerous practical problems associated with application *per os*. More commonly, PUFAs are administered with a selected type of oil. Linseed oil and full-fat linseed are rich sources of PUFAs, in particular ALA (about 50–60% of total FA) and LA (about 13–18% of total FA). For this reason, linseed oil is considered to be one of the most precious sources of ALA from vegetable oils.

Despite the fact that the gastrointestinal tract of horses is adapted to digest a high-fibre diet rather than a high-fat diet, horses are capable of consuming a large amount of fat in the diet. Rations containing up to 230 g/kg DM of vegetable oil (corn or peanut oil) appears to be most eagerly consumed by horses without negatively affecting nutrient digestion (Kronfeld et al., 2004). Furthermore, diets with a decreasing proportion of starch from corn in favour of an increasing proportion of corn oil (5, 10, or 15% of DM) do not negatively affect the digestibility processes (Bush et al., 2001). Similarly, Zeyner et al. (2002), in a longitudinal study on high-fat (soybean oil fed at 1.43 g per kg BW/day) compared to high-starch diet in exercised horses, did not find any significant disadvantage. In contrast, a negative effect of soybean oil (150 g/day/head) addition to the diet on neutral detergent fibre (NDF) and acid detergent fibre (ADF) digestibility has been described (Jansen et al., 2000; 2007). Probably for this reason, the NRC (2007) requirements recommend that soybean oil should be limited to 0.7 g/kg BW/day. In turn, German nutrient requirements for horses (Meyer and Coenen, 2009) do not specify an optimal dose of oil for sport horses, however, they suggest a dose of 1.0–1.5 g of vegetable oil per kg BW/day, but only for soybean, rapeseed, or sunflower oils.

It is worth noting that the negative impact of dietary oil supplementation on digestive function may not only be dose-dependent but may also vary based on the type of oil. Due to its high PUFA content and beneficial omega-6 to omega-3 fatty acid ratio, linseed oil appears to be an exceptionally promising dietary supplement. Unfortunately, the available literature provides limited data on the effects of linseed oil on digestibility processes and their physiological consequences, particularly concerning possible dose-dependent effects. The research hypothesis assumed that the addition of linseed oil to horse feed rations does not reduce the digestibility of nutrients and physiological parameters. Therefore, the aim of this study was to determine the effect of two dietary doses of linseed oil on nutrient apparent digestibility and selected blood parameters in non-working horses.

Material and methods

Experimental design

All experimental procedures were approved by the Local Ethics Committee (Krakow, Poland, Resolution No. 78/2018). The study was conducted at the Przegorzały Experimental Station, which belongs to the University of Agriculture in Krakow. The experiment involved six mares (Konik Polski breed, aged 3 to 12 years) with a BW range of 200–370 kg (mean 323 kg). All horses were clinically healthy, and deworming was conducted before the experiment. The animals were kept in individual

boxes bedded with wood shavings and had *ad libitum* access to water. Regardless of the weather, the animals were turned out daily to a paddock without pasture access from 7:30 to 16:30. There was no health issue or medical intervention during the whole study.

The standard daily ration was calculated for an average BW of 323 kg based on NRC (2007) guidelines for non-working horses (average activity; Table 1). The diets were administered in quantities based on the horse's BW, maintaining a ratio of 1.5 kg DM per 100 kg BW. The horses received a diet consisting of meadow hay (80% DM) and a concentrate mix (20% DM) in the form of pellets.

Table 1. Composition and nutritive value of standard daily rations for horse of BW 323 kg administrated according to real body weight of animals

Item	Group		
	C	Oil150	Oil300
Meadow grass, kg	4.3	4.3	4.3
Concentrate mix, kg	1.1	1.1	1.1
Linseed oil, ml	0.0	150.0	300.0
Nutritive value, % DM			
DM, %	89.1 ± 0.02	89.4 ± 0.05	89.7 ± 0.11
CP	8.8 ± 0.01	8.5 ± 0.06	8.2 ± 0.11
CF	29.9 ± 0.22	29.0 ± 0.17	28.1 ± 0.28
NDF	54.9 ± 0.45	53.1 ± 0.38	51.4 ± 0.41
ADF	36.8 ± 0.25	35.6 ± 0.20	34.5 ± 0.34
EE	2.0 ± 0.01	5.4 ± 0.56	8.2 ± 1.16

DM – dry matter, CP – crude protein, CF – crude fibre, NDF – neutral detergent fibre, ADF – acid detergent fibre, EE – ether extract; C – ration without oil (control), Oil150 – ration with the addition of 150 ml linseed oil/day/horse, Oil300 – ration with the addition of 300 ml linseed oil/day/horse; concentrate mix in pelleted form

The pellets contained oats (32.0% per kg), barley (29.8%), barley straw (24.6%), molasses (6.1%), and a mineral-vitamin supplement (per kg: IU: vitamin A 500 000, vitamin D_3 50 000, vitamin E 6 600; mg: vitamin B_1 330, vitamin B_2 350, vitamin B_6 250, niacin 1000, pantothenic acid 480, folic acid 150 mg, DL-α-tocopherol 6000 mg, choline chloride 6400 mg, and betaine 3430; µg: vitamin B_{12} 1100, biotin 1600; 3%, Horsemix Universal; Dolfos, Piotrków Trybunalski, Poland). Additionally, the animals received 0, 150, or 300 ml of linseed oil per day, amounting to 0.4 to 1.5 ml/day/kg BW. The cold-pressed oil from common linseeds (*Linum usitatissimum* L.) was produced at the Institute of Natural Fibers and Herbal Plants (Poznań, Poland). The chemical composition of the feeds and the fatty acid profile of the linseed oil are presented in Table 2.

The experiment was conducted in a 3 × 3 repeated Latin square design with three groups of animals and three doses of oil (0/150/300 ml of linseed oil/day). The study consisted of three periods of 24 days each, including 21 days of adaptation to the experimental conditions and three days of the trial period. During the trial period, feed intake was monitored, and samples of feed and faeces were collected for chemical analysis each day. Linseed oil was introduced to the diet gradually to adapt the gut microbiota to the composition of the experimental diets. The daily dose was mixed with the concentrate directly before feeding and administered twice daily in equal portions during morning and evening feedings (7:00 and 17:00).

Observations and sampling

Feed intake was monitored daily by weighing the amount of feed offered and refused. Representative feed samples were collected once a week and then combined into a pooled sample for each study period. Digestibility was determined using the marker method with acid-insoluble ash (AIA) as an internal marker, according to the method described by Van Keulen and Young (1977). At the beginning and end of each period, the BW of the horses was measured using a zootechnical scale. Faecal structure was evaluated daily according to a 5-point scale of fluidity (1 – osmotic liquid diarrhoea, 4 – normal manure, 5 – very dry small faecal balls; Johnson and Rossow, 2018).

Faecal samples were collected rectally during the last three days of each period and stored at −20 °C until chemical analyses. From day 22 through day 24 of each period, twelve faecal samples were collected at 06:00, 12:00, 18:00, and

Table 2. Nutritive value of feeds (% DM) and linseed oil fatty acids profile (% of total fatty acids)

Item	DM, %	Ash	CP	EE	CF	NDF	ADF	ADL	Starch	NSC	GE, kcal/g DM
Meadow hay	90.7	7.1	8.5	1.9	33.9	60.9	41.7	6.2	–	–	4285
Concentrate mix	90.6	10.1	9.7	2.5	13.5	31.4	18.3	2.2	38.1	4.9	4220
Linseed oil	C 14	C 16	C 16:1 n7	C 18	C 18:1 n9	C 18:2 n6	C 18:3 n3	EFA	MUFA	PUFA	n6/n3
	0.2	7.4	0.1	3.0	19.7	14.6	54.7	10.7	19.8	69.3	0.2:1

DM – dry matter, ash – crude ash, CP – crude protein, EE – ether extract, CF – crude fibre, NDF – neutral detergent fibre, ADF – acid detergent fibre, NSC – non-structural carbohydrates, GE – gross energy, EFA – essential fatty acids, MUFA – monounsaturated fatty acids, PUFA – polyunsaturated fatty acids; concentrate mix in pelleted form; for linseed oil % in total fatty acid; n6/n3 – ratio of n-6 to n-3 fatty acids

00:00 on day 22; at 04:00, 10:00, 16:00, and 22:00 on day 23; and at 02:00, 08:00, 14:00, and 20:00 on day 24, ensuring that every 2-h interval of the 24-h feeding cycle was represented.

Gas production test

Additionally, an *in vitro* gas production (GP) experiment was conducted to better understand the reasons for differences in nutrient digestion resulting from varying levels of linseed oil supplementation. For this purpose, a separate trial was carried out using faecal inoculum from horses fed with or without linseed oil supplementation to determine the GP and organic matter digestibility (OMD) coefficients of two samples differing in starch and fibre content (meadow hay and oat grain). The test assumed that GP results would depend not only on the chemical composition of the fermented material but also on the composition of the faecal microbiota, which could be influenced by dietary linseed oil supplementation. Estimation of total gas production (TGP) and OMD of the two experimental feed samples (meadow hay and oat grain) was performed on the last day of each trial period. Faecal samples were collected from the rectum of each animal at 8:00 and then transported in separate thermos flasks to the laboratory, where the GP test was carried out according to the method described by Menke and Steingas (1988).

Prior to placing feces in the thermoses, they were preheated with hot water to 38 °C. After sample collection, the free spaces in thermoses were filled with CO_2 for 30 s before closing. At the laboratory, 50 g of faeces from each individual in the same experimental group were mixed together under anaerobic conditions for 30 s to obtain a homogeneous inoculum. Then, the biological material was mixed with buffer according to the method proposed by Menke and Steingas (1988) in a faeces:buffer ratio of 1:3. The buffered inoculum (30 ml) was filled into 250-ml glass syringes along with pre-weighed samples of meadow hay or oat grain (0.2 g). Prior to weighing, feed samples were ground to a sieve size of 0.75 mm using a Pulverisette Laboratory Cutting Mill (Fritsch GmbH, Idar-Oberstein, Germany). For each feed type and each group of horses, six syringes (replicates) and two blanks were prepared for each experimental period. As the digestion experiment consisted of three separate periods (3 × 3 Latin square), the total number of replicates per treatment and feed sample was 18 (n = 18). After filling, the syringes were incubated in a water bath with a shaker at 39.0 °C for 48 h. Total gas production was measured after 2, 4, 6, 24, and 48 h of incubation, and net TGP (ml/g DM) was calculated by subtracting the gas produced in the blanks. Net TGP after 24 h of incubation (TGP24) was used to calculate OMD according to the formula by Menke et al. (1979):

OMD (%) = 14.88 + 0.889 TGP24 (ml/200 mg DM) + 0.45 CP (%) + 0.065 Ash (%).

Blood collection and analysis

Blood samples were taken from the jugular vein of the horses on the last day of each data collection period at three time points: before feeding (0), and 2 and 4 h after a meal. Samples for blood count were collected into tubes containing potassium EDTA (1.6 mg EDTA/ml of blood; Sarstedt, Germany) and delivered immediately to a commercial laboratory (IDEXX Laboratory, Warsaw, Poland). Haematological analyses were performed using the ProCyte Dx Analyzer (IDEXX Laboratory) and included white blood cell (WBC) count, red blood cell (RBC) count, haemoglobin (HGB) concentration, packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), and PLT count. Additionally, 10 ml of blood samples were collected separately into tubes with a clot activator (Serum Separator 18203; Kima, Piove di Sacco, Italy), centrifuged at 1800 *g* for 10 min using a Centrifuge MPW-223e (MPW Instruments, Warsaw, Poland). Separated serum was immediately chilled to +4 °C and delivered to the commercial laboratory (IDEXX Laboratory). Serum was analyzed for urea, creatinine, Na, K, bilirubin, alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), total protein (TP), albumin, glucose, cholesterol, and triglycerides using the Catalyst One apparatus (IDEXX Laboratory).

Chemical analysis of feeds and faeces

Dry matter, ash, crude protein (CP), ether extract (EE), and crude fibre (CF) were determined using standard analytical procedures (Methods No. 934.01, 942.05, 976.05, 920.39, and 978.10, respectively; AOAC International, 2007). Starch content in the concentrate mix was determined by the enzymatic method (Faisant et al., 1995). Water-soluble carbohydrates (WSC) were analysed using the anthrone method (Dubois et al., 1956). NDF, ADF, and acid detergent lignin (ADL) were analysed using the Ankom220 Fiber Analyzer (Ankom Technology, Macedon, NY, USA) according to Mertens (2002), AOAC International (2007; method 973.18), and Robertson and Van Soest (1981), respectively. Gross energy (GE) was determined using a bomb calorimeter (KL-10; Precyzja-Bit, Bydgoszcz,

Poland). The fatty acids profile of linseed oil was analysed using a Varian 450-GC gas chromatograph (Varian, Palo Alto, CA, USA) equipped with an Rt-2560 capillary column (100 m × 0.25 mm × 0.20 μm) (Restek Corporation, Bellefonte, PA, USA). Moreover, feeds and faeces were analysed for AIA according to Van Keulen and Young (1977).

Calculations and statistical analysis

The data were statistically analysed using the GLIMMIX procedure of the SAS statistical package (version 9.4, SAS Institute). For blood parameters, the statistical model included the effects of experimental group (C, Oil150, and Oil300), blood collection time relative to morning feeding (0, 2, and 4 h), and their interactions between these effects as categorizing variables. The effects of the animal (n = 6) and the experimental period (n = 3) were included in the statistical model as random variables. No significant changes in the BW of the animals were observed throughout the experiment thus this parameter was excluded from the analyses. For nutrient digestibility parameters, the statistical model included the effect of the treatment (n = 3) as a categorizing variable, while the effect of the square, animal within square, and experimental period were included as random variables. Repeatability of measurements on a single subject was accounted for by introducing the residual option as a random variable. The experimental subject was considered to be the animal. In the case of a significant effect of blood collection time and/or interactions between the experimental group effect and blood collection time effect, means were separated using Tukey's test. Prior to analysis, the normal distribution of the data was checked using the Shapiro-Wilk test, and if necessary, data fitting to the model was improved using the lognormal option. The research hypotheses for blood parameters and nutrient digestibility indices were also verified based on planned contrasts allowing comparison of differences in the studied parameters between individual experimental groups (C vs. Oil150 and Oil300; Oil150 vs. Oil300). Results are presented in tables as least squares mean plus standard errors of the mean (SEM). Effects between experimental factors were considered statistically significant at $P \leq 0.05$, and trends were noted when $0.05 < P \leq 0.10$. Results from the *in vitro* gas production experiment were analysed using one-way analysis of variance and the Kruskal-Wallis test in R software. The statistical model included the effect of the experimental group as a categorising variable, while the effect of the experimental period was included in the statistical model as a random variable.

Results

Except for EE, no significant effect of dietary linseed oil supplementation on *in vivo* nutrient digestibility was observed (Table 3). Compared to the C group, an increase in the EE coefficient was noted by 15.26% in the Oil150 group and by 18.63% in the Oil300 group ($P < 0.001$).

Table 3. Effect of linseed oil addition to the diet for non-working horses (n = 6, 3 × 3 Latin square design) on nutrient digestibility coefficients (%)

Nutrient	Group			SEM	Effect of group	Contrasts	
	C	Oil150	Oil300			C vs. Oil150 and Oil300	Oil150 vs. Oil300
DM	72.1	73.0	73.5	2.24	0.940	0.750	0.930
OM	73.6	74.4	75.0	2.63	0.930	0.750	0.910
CP	77.7	75.6	77.9	1.46	0.430	0.600	0.220
EE	69.7	85.0	88.4	1.90	<0.001	<0.001	0.190
CF	60.6	61.3	60.4	3.69	0.900	0.880	0.700
NDF	62.0	62.1	62.0	3.25	0.970	0.850	0.920
ADF	56.9	58.3	58.2	3.73	0.980	0.860	0.910

DM – dry matter, OM – organic matter, CP – crude protein, EE – ether extract, CF – crude fibre, NDF – neutral detergent fibre, ADF – acid detergent fibre, SEM – standard error of the mean; C – control group (without oil), Oil 150 – diet with 150 ml linseed oil/day/animal, Oil 300 – diet with 300 ml linseed oil/day/animal; $P < 0.05$ means that the data is significantly different

Regarding the results from *in vitro* GP studies (Table 4), it was found that the use of inoculum from animals fed diets with an increased proportion of linseed oil resulted in a tendency ($P = 0.070$) to decrease TGP (from 20.38 to 14.40 ml/g DM) of the sample containing high-fibre meadow hay.

Regardless of the experimental period or diet type, horse faeces were characterized by a normal structure (3.9–4.0 points) and were not analysed statistically.

Table 4. Total net gas production (TGP) and *in vitro* organic matter digestibility (IVOMD) of meadow hay and oat grain depending on inoculum source

Feed	Parameter	Group			SEM	*P*
		C	Oil150	Oil300		
Meadow hay	TGP, ml/g DM	20.4	18.8	14.4	1.13	0.070
n = 18	IVOMD, %	33.2	32.8	29.5	0.98	0.320
Oat grain	TGP, ml/g DM	36.4	38.5	34.7	0.45	0.640
n = 18	IVOMD, %	52.4	55.9	51.8	0.51	0.580

DM – dry matter, SEM – standard error of the mean; C – control group (without oil), Oil150 – diet with 150 ml linseed oil/day/animal, Oil300 – diet with 300 ml linseed oil/day/animal; $P < 0.05$ means that the data is significantly different

The addition of oil to the horse diet did not affect most of the analysed blood morphological parameters (Table 5). A significant treatment effect

Table 5. Effect of linseed oil addition to the diet for non-working horses (n = 6, 3 × 3 Latin square design) on blood morphological parameters

Parameter	T, h	Group			SEM	Main effect			Contrasts	
		C	Oil150	Oil300		G	T	G × T	C vs. Oil150 and Oil300	Oil150 vs. Oil300
WBC, G/l	0	8.6^{B}	8.5^{B}	8.6^{B}	0.46	0.70	0.006	0.960	0.450	0.740
	2 h	8.8^{B}	8.7^{B}	8.6^{B}						
	4 h	9.3^{A}	9.0^{A}	9.3^{A}						
RBC, T/l	0	8.5^{B}	8.3^{B}	8.5^{B}	0.49	0.240	<0.001	0.840	0.820	0.100
	2 h	9.3^{A}	9.3^{A}	9.4^{A}						
	4 h	9.1^{A}	9.0^{A}	9.6^{A}						
HGB, g/dl	0	13.1^{B}	12.8^{B}	13.2^{B}	0.65	0.100	<0.001	0.860	0.870	0.030
	2 h	14.5^{A}	14.4^{A}	14.8^{A}						
	4 h	14.3^{A}	13.8^{A}	14.9^{A}						
PCV, %	0	34.8^{B}	33.8^{B}	35.0^{B}	1.79	0.100	<0.001	0.800	0.930	0.030
	2 h	37.8^{A}	37.8^{A}	38.7^{A}						
	4 h	37.3^{A}	36.2^{A}	38.8^{A}						
MCV, fl	0	41.7^{A}	41.7^{A}	41.7^{A}	2.50	0.440	0.006	0.500	0.200	1.000
	2 h	41.3^{AB}	41.3^{AB}	41.3^{AB}						
	4 h	41.5^{B}	41.0^{B}	41.0^{B}						
MCH, pg	0	15.8	15.8	15.7	0.89	0.820	0.450	0.730	0.530	1.000
	2 h	15.8	15.7	15.8						
	4 h	15.7	15.7	15.7						
MCHC, g/dl	0	37.5^{B}	37.7^{B}	37.8^{B}	0.44	0.390	0.001	0.790	0.170	1.000
	2 h	38.3^{A}	38.3^{A}	38.3^{A}						
	4 h	38.0^{A}	38.5^{A}	38.3^{A}						
PLT, G/l	0	166.0	162.4	150.7	13.51	0.009	0.740	0.670	0.180	0.006
	2 h	163.2	170.7	154.5						
	4 h	163.7	162.5	156.7						

WBC – white blood cells, RBC – red blood cells, HGB – haemoglobin content, PCV – packed cell volume, MCV – mean corpuscular volume, MCH – mean corpuscular haemoglobin, MCHC – mean corpuscular haemoglobin concentration, PLT – platelet count, T – sampling time: 0, 2 h and 4 h after a meal, G – group, SEM – standard error of the mean; C – control group (without oil), Oil150 – diet with 150 ml linseed oil/day/animal, Oil300 – diet with 300 ml linseed oil/day/animal; ^{AB} – means in the column marked with different capital letters are significantly different at $P \leq 0.05$ for T

was observed only for PLT, where animals receiving a smaller amount of oil (Oil150 vs. Oil300) had an increased concentration of PLT ($P = 0.009$). Regardless of the time of collection, a trend towards decreased HGB and PCV, compared to the C group ($P = 0.100$), was observed with 150 ml oil supplementation. In contrast, the 300 ml dose significantly increased these parameters (Oil150 vs. Oil300, $P = 0.030$). A significant effect of blood sampling time (0, 2, or 4 h after a meal; $P < 0.01$) on all measured indicators, except MCH and PLT, was observed. Generally, as time passed from feed administration, their values constantly increased.

The effects of linseed oil addition to the diet on TP, albumin, urea, bilirubin, creatinine, and glucose concentration in the blood are summarised in Table 6, and on AST, ALT, ALP, cholesterol, and triglycerides in Table 7. Significant treatment effects were observed for TP ($P = 0.003$), albumin ($P = 0.030$), urea ($P = 0.004$), bilirubin ($P = 0.020$), AST ($P < 0.001$), ALT ($P = 0.010$), ALP ($P < 0.001$), and cholesterol ($P < 0.001$). An effect of increasing oil proportion in the diet on TP and albumin concentration was observed (Oil150 vs. Oil300, $P \leq 0.001$ and $P = 0.01$, respectively). For urea, ALT, and ALP, the effect of oil addition to the diet was noted regardless of its amount (C vs. Oil150 and Oil300, $P \leq 0.001$). Both the addition of the oil and its amount significantly influenced bilirubin, AST, and cholesterol. There was a trend towards higher bilirubin concentration in the blood of animals receiving oil supplementation (C vs. Oil150 and Oil300, $P = 0.070$), as well as an increase in its level with increasing oil amount in the diet (Oil150 vs. Oil300, $P = 0.030$). Similarly, AST and cholesterol concentrations increased both due to oil supplementation (C vs. Oil150 and Oil300, $P < 0.001$) and with the increasing proportion of oil in the diet (Oil150 vs. Oil300, $P < 0.001$). All analysed parameters, except triglycerides, were affected by blood sampling time ($P \leq 0.02$). In samples taken 2 h after feeding, these parameters increased compared to baseline (T0), then their values slightly decreased.

Table 6. Effect of linseed oil addition to the diet for non-working horses (n = 6, 3 × 3 Latin square design) on total protein, albumin, urea, bilirubin, creatinine, and glucose content in blood

Parameter	T, h	Group			SEM	Main effect			Contrasts	
		C	Oil150	Oil300		G	T	G × T	C vs. Oil150 and Oil300	Oil150 vs. Oil300
Total protein, g/l	0	62.8^{C}	60.7^{C}	64.5^{C}	2.34	0.003	<0.001	0.460	0.350	<0.001
	2 h	69.2^{A}	70.8^{A}	73.0^{A}						
	4 h	67.2^{B}	65.0^{B}	69.0^{B}						
Albumin, g/l	0	28.3^{C}	27.5^{C}	28.2^{C}	0.84	0.030	<0.001	0.730	0.800	0.010
	2 h	32.8^{A}	32.3^{A}	33.5^{A}						
	4 h	30.7^{B}	29.7^{B}	31.8^{B}						
Urea, mmol/l	0	5.2^{B}	4.7^{B}	4.8^{B}	0.31	0.004	0.020	0.830	<0.001	0.730
	2 h	5.4^{A}	5.2^{A}	5.1^{A}						
	4 h	5.3^{AB}	5.0^{AB}	4.9^{AB}						
Bilirubin, µmol/l	0	16.1^{B}	15.9^{B}	16.8^{B}	0.87	0.020	<0.001	0.870	0.070	0.030
	2 h	17.8^{A}	18.3^{A}	18.7^{A}						
	4 h	15.7^{B}	15.9^{B}	17.1^{B}						
Creatinine, µmol/l	0	75.3^{B}	74.5^{B}	76.0^{B}	5.30	0.840	<0.001	0.210	0.770	0.610
	2 h	81.7^{A}	85.2^{A}	83.5^{A}						
	4 h	80.5^{B}	75.7^{B}	78.0^{B}						
Glucose, mmol/l	0	4.8^{B}	5.0^{B}	5.1^{B}	0.38	0.590	0.002	0.760	0.470	0.460
	2 h	6.0^{A}	6.0^{A}	5.7^{A}						
	4 h	5.0^{B}	5.6^{B}	5.2^{B}						

T – sampling time: 0, 2 h and 4 h after a meal, G – group, SEM – standard error of the mean; C – control group (without oil), Oil150 – diet with 150 ml linseed oil/day/animal, Oil300 – diet with 300 ml linseed oil/day/animal; ^{ABC} – means in the column marked with different capital letters are significantly different at $P \leq 0.05$ for T

Table 7. Effect of linseed oil addition to the diet for non-working horses (n = 6, 3 × 3 Latin square design) on AST, ALT, alkaline phosphatase, cholesterol, and triglycerides content in blood

Parameter	T, h	Group			SEM	Main effect			Contrasts	
		C	Oil150	Oil300		G	T	G × T	C vs. Oil150 and Oil300	Oil150 vs. Oil300
	0	399.5^{C}	406.8^{C}	438.7^{C}						
AST, U/l	2 h	434.0^{A}	468.2^{A}	503.3^{A}	18.68	<0.001	<0.001	0.330	<0.001	<0.001
	4 h	430.0^{B}	435.5^{B}	464.7^{B}						
	0	10.7^{B}	11.2^{B}	10.8^{B}						
ALT, U/l	2 h	11.3^{A}	13.0^{A}	13.0^{A}	0.86	0.010	<0.001	0.230	0.003	0.730
	4 h	10.7^{B}	11.0^{B}	11.7^{B}						
	0	189.3^{C}	203.8^{C}	212.0^{C}						
ALP, U/l	2 h	205.0^{A}	230.0^{A}	238.0^{A}	24.23	<0.001	<0.001	0.630	<0.001	0.150
	4 h	201.5^{B}	219.2^{B}	220.7^{B}						
	0	2.5^{C}	2.7^{C}	3.0^{C}						
Cholesterol, mmol/l	2 h	2.7^{A}	3.2^{A}	3.3^{A}	0.11	<0.001	<0.001	0.220	<0.001	<0.001
	4 h	2.6^{B}	2.9^{B}	3.1^{B}						
	0	0.23	0.25	0.25						
Triglycerides, mmol/l	2 h	0.23	0.27	0.22	0.050	0.160	0.820	0.320	0.060	0.680
	4 h	0.18	0.28	0.30						

AST – aspartate aminotransferase, ALT – alanine aminotransferase, ALP – alkaline phosphatase, T – sampling time: 0, 2 h and 4 h after a meal, G – group, SEM – standard error of the mean; C – control group (without oil), Oil150 – diet with 150 ml linseed oil/day/animal, Oil300 – diet with 300 ml linseed oil/day/animal; ^{ABC} – means in the column marked with different capital letters are significantly different at $P \leq 0.05$ for T

Regardless of the amount used, the addition of oil to the diet did not affect Na and K concentration in the blood (Table 8). However, a significant effect of blood sampling time was observed for Na ($P < 0.01$), with concentration increased 2 h after feeding, then decreased. An opposite trend was observed for K levels in the blood.

Table 8. Effect of linseed oil addition to the diet for non-working horses (n = 6, 3 × 3 Latin square design) on the blood sodium and potassium content

Parameter	T, h	Group			SEM	Main effect			Contrasts	
		C	Oil150	Oil300		G	T	G × T	C vs. Oil150 and Oil300	Oil150 vs. Oil300
Sodium, mmol/l	0	140.8^{B}	140.5^{B}	141.8^{B}	1.15	0.370	<0.001	0.230	0.830	0.160
	2 h	143.0^{A}	143.8^{A}	145.7^{A}						
	4 h	141.0^{B}	139.3^{B}	139.3^{B}						
Potassium, mmol/l	0	3.9^{A}	4.5^{A}	4.5^{A}	0.40	0.450	<0.001	0.900	0.260	0.570
	2 h	3.3^{B}	3.4^{B}	3.3^{B}						
	4 h	4.9^{A}	5.0^{A}	4.9^{A}						

T – sampling time: 0, 2 h and 4 h after a meal, G – group, SEM – standard error of the mean; C – control group (without oil), Oil150 – diet with 150 ml linseed oil/day/animal, Oil300 – diet with 300 ml linseed oil/day/animal; ^{AB} – means in the column marked with different capital letters are significantly different at $P \leq 0.05$ for T

Discussion

The adverse effects of high concentrations of rapidly degradable carbohydrates in the daily ration of horses are well-known and are increasingly mitigated in practice by incorporating fat in their diets. Oil is a commonly used energy source for animals, enhancing the energy density of the ration, which is crucial, especially for performance horses with high energy demands (Kronfeld, 1996). In the present study, we hypothesised that appropriate fat supplementation in a horse's diet might not negatively affect nutrient digestibility or physiological parameters. Feeds rich in fat-derived energy also help to reduce DM intake, positively affecting the condition of sport horses (Harris, 1997; Kronfeld and Harris, 2003). This is particularly important for limiting fluctuations in the daily BW of such animals, which depend mainly on feed and water intake, frequency of defecation, and body dehydration during exercise. In some cases, BW can vary by up to 5 kg per day (Webb and Weaver, 1979). For example, horses on pasture can increase their BW by up to 5% in a single day (Dugdale et al., 2011). When additional fat was supplemented to the ration (1.43 g/kg BW/day) compared to standard feeding, it was observed that horses gained BW quickly, especially with prolonged feeding (Zeyner et al., 2002; Godoi et al., 2010). During our experiment, supplementing 300 ml of oil per horse per day resulted in a 4.8% BW increase within 24 days, with maximum fluctuations of ±17 kg. Notably, faecal consistency remained unchanged throughout the experiment, indicating no negative impact of the oil on digestive processes.

In this context, it is worth noting that linseed oil not only provides energy but also n-3 EFAs which have health-promoting properties. These EFAs contribute to the formation of anti-inflammatory molecules called resolvins, inhibit pro-inflammatory cytokines production, and serve as precursors for many essential metabolites. Moreover, EFAs play a role in cholesterol transport and oxidation and help build cell membranes (Dyerberg et al., 1975; Das, 2006; Calder, 2008; Frota et al., 2010). Additionally, linseed oil contains approximately 12.7–14.5% LA and 53.3–54.7% ALA, resulting in an n-6/n-3 ratio of 0.2:1 (Warren and Vineyard, 2013), which, represents the desired ratio.

Horses, as herbivores, are not evolutionarily adapted to high-fat intake, but some studies shown that their fat digestibility can reach 90% (Kronfeld et al., 2004; Meyer and Coenen, 2009). High-fat diets increase bile production, thereby enhancing fat digestibility (Jansen et al., 2000). Horses prefer vegetable oils over animal-based fats due to better palatability (Holland et al., 1998). In our study, even at a dose of 300 ml/animal/day, linseed oil did not affect nutrient digestibility except for EE. The digestibility of EE increased with higher oil doses, from 69.7% in the control (C) group to 88.4% in the Oil300 group. A similar effect was observed by Julen et al. (1995) in Quarter Horses in training, where EE digestibility increased from 40.5% to 70.7% when a concentrate containing 10% EE of animal origin was fed to the experimental group. Similarly, studies on Thoroughbreds receiving 9.6% EE in their diet (compared to 2.5% EE in the control group) showed no reduction in nutrient digestibility (Hughes et al., 2010). Kronfeld et al. (2004) also found that diets containing up to 230 g of EE per kg DM (using corn oil, peanut oil, or a vegetable-animal oil mix) had no negative impact on nutrient digestibility. These authors also suggest that optimal EE digestibility can be achieved when a ration containing about 100–150 g EE/kg DM is fed. Saastamoinen and Särkijärvi (2020) conducted a study examining the impact of linseed groat-based feed supplements on nutrient digestibility and

blood parameters in horses. They found that diets supplemented with linseed groats significantly improved the digestibility of CP and EE compared to a control diet. Importantly, no adverse effects on blood parameters were observed, suggesting that linseed supplementation was safe and beneficial for improving nutrient absorption in horses.

We acknowledge that our study has certain limitations, particularly those related to the use of AIA as an internal marker, as total faecal collection was not feasible. AIA method, while widely used in digestibility trials with horses, has known constraints, particularly in terms of marker recovery and potential variability between individuals.

The research results presented above were obtained using various horse breeds, which may have potentially affected digestibility coefficients. Primitive breeds, including Polish Konik used in the current research, are often referred to as "easy keepers", more effectively digest and absorb nutrients from feed and are more prone to BW gain, obesity, and metabolic diseases like metabolic syndrome and insulin resistance than warmblood horses (Frank, 2011). Comparative studies among breeds (pony, American Trotter, Andalusian) with diets based on fibrous, grain-rich, or high-fat feeds for 20 weeks showed no breed influence on nutrient digestibility (Potter et al., 2022). However, in this study, digestibility coefficients were significantly affected by nutrient intake, with higher EE content in diets generally accompanied by higher EE digestibility. On the other hand, more EE in the diet increases its flow to the caecum, possibly limiting cellulolytic microbial activity and fibre utilisation, as seen in ruminants fed diets containing over 50 g fat/kg DM (Brooks et al., 1954). In horses, reduced NDF and ADF digestibility has been observed when soybean oil, in quantities ranging from 100 to 283 g/day, was fed (Jansen et al., 2000; 2001; 2002). However, this reduction was only found with soybean oil, and its underlying mechanism remains unclear.

In vitro digestibility trial

In vivo digestibility studies are the most reliable reflection of actual digestive processes, but they are labor-intensive, lengthy, and costly. *In vitro* studies provide a quicker assessment of the nutritive value of feeds without directly using experimental animals. The gas-test technique is particularly useful, constantly measuring gas release during *in vitro* sample fermentation to determine feed fermentation rate and extent, which reflects processes occurring in the animal's digestive tract (Menke and Steingass, 1988). Initially, rumen fluid was used as a microorganism source (inoculum) for fermentation studies, currently, faecal inoculum – collected less invasively from both ruminants (Akhter et al., 1999) and equines (Lowman, 1998) – is widely applied. The main limitation of the *in vitro* gas production technique is that it gives only a simplified estimation of microbial fermentation and cannot fully reproduce the complexity of the equine hindgut ecosystem, especially because we used faecal inoculum. In our study, compared to the control group, the use of faecal inoculum taken from horses fed linseed oil showed a tendency toward lower TGP (20.38% vs. 14.40%) and numerically lower IVOMD of fibrous meadow hay. This relationship was dose-dependent, meaning that higher oil doses in the diet resulted in greater restrictions in gas production. A similar pattern was not observed for starch-rich oat grain. These results suggest that adding linseed oil to the diet may alter gut microbiota composition or the activity of specific microorganisms, which in our study was confirmed by slight numerical differences in fermentation parameters. The reduced TGP of high-fibre hay may indicate a progressive decrease in cellulolytic microbial activity with increasing proportions of linseed oil in the diet. These observations are consistent with studies showing reduced *in vivo* fibre digestibility with dietary soybean oil supplementation exceeding 100 g/day (Jansen et al., 2002). However, we believe that in this particular case, the fatty acid composition of the tested oils, especially the high ALA content in linseed oil, may play a significant role in animal health, due to, e.g., its anti-inflammatory properties. Unfortunately, due to the limited number of similar studies in the available literature, it is difficult to compare our results with those of other studies using linseed oil. Regardless of the inoculum source, the range of our *in vitro* test results was comparable to those obtained in the study by Macheboeuf et al. (1998), where the gas-test method was used to predict the OMD of different forages in horses.

Blood parameters

In the present study, increasing dietary linseed oil supplementation from 150 to 300 ml/animal/day caused a decrease in PLT concentration in the blood without significant changes in blood morphology. However, the increase in oil in the diet affected some blood biochemical parameters, raising total protein, albumin, bilirubin, AST, and cholesterol levels while reducing ALT and ALP activities compared to the control group. The increase in blood cholesterol in exercised horses receiving oil has been observed previously by

Hansen et al. (2002) and Zeyner et al. (2002), who also used dietary supplementation with vegetable fats.

Our study showed an increase in blood morphological values was observed over time after feed intake, except for MCH and PLT, which was similar to the findings of Zeyner et al. (2002) in a 390-day study with 20 warmblood horses in regular training fed soybean oil. These authors found increased blood glucose, TP, and albumin concentrations when animals received a high-fat diet compared to those on a high-starch diet. Patoux and Istasse (2016) studied the effects of dietary linseed oil and sunflower oil in horse diet on haematology and fatty acids profiles in red blood cell membranes. The results showed that both oils supplementation made an increased the concentrations of RBC, HGB, haematocrit and reduced platelet concentrations by approximately 13%, while still remaining within physiologically acceptable ranges. In a similar context involving dietary linseed oil supplementation in horses, Hansen et al. (2002) reported that supplementation decreased platelet aggregation in response to inflammation. Likewise, linseed oil and marine fatty acid supplementation reduced platelet aggregation in humans and dogs (Casali et al., 1986; Allman et al., 1995).

In turn, Sembratowicz et al. (2020) conducted a study comparing the effects of soybean oil and linseed oil on the biochemical and haematological parameters, as well as the redox status of horse blood. Their results showed that horses receiving linseed oil had significantly lower plasma levels of glucose, low-density lipoprotein cholesterol, total cholesterol/high-density lipoprotein ratio and triacylglycerols. Moreover, they showed an increase in red blood cell indicators, lymphocyte count, lysozyme levels, and the activities of ALT, ALP, superoxide dismutase, and catalase.

Supplementation with linseed oil significantly improved the lipid profile in blood, haematological parameters, and antioxidant defence mechanisms, suggesting health benefits from replacing soybean oil with linseed oil in horse diets. It is also worth noting that in our experiment, bilirubin concentration was influenced more by the time elapsed since feed intake rather than diet composition. There was no effect of adding linseed oil to the feed on the concentration of sodium and potassium in the blood. Similarly, Hoyt et al. (1995) found no changes in sweat Na levels in horses fed high- or low-fat diets, which may be important because study from 1996 suggest that fat supplemented diet reduced daily heat load by 5% and water requirement by 12% (Kronfeld, 1996).

Conclusions

In conclusion, the results of this study showed that dietary linseed oil supplementation enhances energy intake in horses without any negative effects on nutrient digestion or physiological blood parameters. The addition of oil increases the amount of fat in the horses' diet, which helps improves digestibility of this nutrient. The high digestibility coefficient of fat ensures good utilization of linseed oil as an energy source, which may be particularly important in feeding sport horses, emaciated horses, or those with low weight gain. This may also indicate that the efficiency of digestive processes in horses receiving such a supplement may be modulated directly through increased fat supply or indirectly through the influence of fat on the composition or activity of the gastrointestinal microbiota. Moreover, linseed oil is an excellent source of n-3 fatty acids, providing anti-inflammatory benefits, supporting cell membrane formation, and facilitates cholesterol transport. The effects of adding linseed oil to the horse's diet on exercise capacity, temperament, or coat quality requires further research.

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Conflict of interest

The Authors declare that there is no conflict of interest.

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