



## Exogenous fibrolytic enzymes improve carbohydrate digestion in exercising horses

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**KEY WORDS:** digestibility, enzymes, horses, nutrition, physical activity

Received: 3 June 2019

Revised: 20 December 2019

Accepted: 5 March 2020

**ABSTRACT.** The aim of the study was to investigate the effects of dietary enzymes and training on carbohydrate digestibility, blood morphology and biochemistry in horses. A group of 10 animals was divided into two treatments: control (C) and supplemented with enzymes (ES). For 14 days, group C was fed a diet based on 6 kg of hay and 6 kg of whole grains of oat, while the ES group was fed the same diet with the addition of xylanase/cellulase. After 14 days of the experimental period and 5 days of the washout process, the treatments were reversed for another 14 days. During the whole experiment, horses were trained 6 days a week including walk, trot and gallop. Blood and faeces sample were analysed. Blood parameters were measured before and after training. Sieve analysis of ES horse faeces showed fewer large particles in comparison to C animals. Neutral detergent fibre digestibility was higher in ES horses. Starch digestibility was also elevated in ES animals. Enzyme supplementation did not affect blood parameters or most of the biochemical blood indices, although reductions in blood cholesterol and urea concentrations were noted in ES horses. Leptin concentration was increased and obestatin level was decreased in ES horses. Training increased the number of erythrocytes and leukocytes, haematocrit value and haemoglobin concentration. In addition, it influenced the factors responsible for anabolic/catabolic pathways and turnover of carbohydrates, lipids and proteins, including a decrease in blood insulin level. The study showed that dietary supplementation with fibrolytic enzymes improves feed digestibility and energy balance in horses, and has no visible side effects during exercise.

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### Introduction

Due to the activity of the endogenous microbiota, non-ruminant herbivores can effectively ferment the structural carbohydrate fractions in the hindgut, mainly in the caecum. Traditionally, horse nutrition is based on the use of roughage, which reflects the natural diet of this species. However, working horses

often need additional energy that can be provided with the use of grains, often processed to a certain extent, or fat supplementation. Oats have been widely used as an energy-rich feedstuff. Studies have shown that even though oat starch is better digestible than barley or maize (Kienzle et al., 1997), the energy density of this cereal is relatively low due to its higher fibre content. Thus, meeting the energy requirements

of high performance horses can be difficult if only oats are used. One solution is to provide horses with easily accessible, heavily processed starch but this can lead to rapid fermentation and acidosis of the caecum. Since oat starch is already well digested, increasing its digestibility by thermal processing obviously involves risks. However, targeting its fibrous component may be beneficial (Särkijärvi and Saastamoinen, 2006; Milinovich et al., 2007). An alternative method of improving carbohydrate utilization in horses may be the supplementation of fibrous diet with exogenous fibrolytic enzymes. The usage of these feed additives is a common practice in many non-ruminant species (Masey O'Neill et al., 2014). Moreover, even in ruminant nutrition, fibre-degrading enzymes have been seen to fill the gap between potential and actual animal performance, which is connected with improved cell wall digestion and feed efficiency (Beauchemin et al., 2003). There is insufficient knowledge on the use of exogenous enzymes in horse nutrition and it is often assumed that due to the presence of the caecum, there is little opportunity for the use of such additives. Administration of cellulase to the diets of Arabian geldings fed a cereal based ration actually decreased dry matter intake and fibre digestibility (O'Connor-Robison et al., 2007). In contrast it was demonstrated that horse diet supplementation with a solid-state fermentation enzyme product improved carbohydrate digestion (Hainze et al., 2003). However, in both of these studies, horses were not trained, so the possible effect of physical activity on enzyme supplementation is unknown. Based on research conducted on other non-ruminants, the secondary effects of fibrolytic enzyme supplementation include modulation of gastrointestinal tract (GIT) microbiota, changes in the histomorphometry of the mucosa and in the metabolism of many nutrients (Bedford and Schulze, 1998; Gao et al., 2007; Kuo et al., 2011; Singh et al., 2012; Masey O'Neill et al., 2014). However, there are no studies on the effect of fibrolytic enzymes on the above-mentioned parameters in horses. Therefore, the aim of the present study was to investigate the effects of digestive enzymes and training on the digestibility of carbohydrates as well as blood morphology and biochemistry, including some of the major hormones involved in regulating feed intake.

## Material and methods

### Ethics statement

The study was carried out in strict accordance with the recommendations of the National Ethics Commission (Warsaw, Poland). All procedures and

experiments complied with the guidelines of the Local Ethics Commission of the Poznań University of Life Sciences (Poznań, Poland) with respect to animal experimentation and care of animals under study, and all efforts were made to minimize suffering. For all procedures used in the experiment (training, faecal sampling, blood collection) no permit is needed according to current legislations in Poland (Act of 15 January 2015 on the protection of animals used for scientific or educational purposes).

### Animals and study design

The study was conducted on 10 Belgian Warmblood horses (BWP – Belgisch Warmbloed Paard), including 6 mares and 4 geldings. All horses were 5 years old, their average weight was  $570 \pm 18.2$  kg, and their height at the withers was between 166 and 170 cm. The animals were selected and weighed one week prior the test (WPT/4I 2000H2, RADWAG, Radom) to ensure homogeneity within treatments.

The experiment was carried out in a two-period crossover design. Horses were housed in individual  $3.2 \times 4.0$  m box stalls bedded with wheat straw. Before the experiment horses were randomly, but with equal sex ratio, assigned to control (C;  $n = 5$ ) or enzyme supplemented (ES;  $n = 5$ ) groups. The experimental design is given in Figure 1. Entire experimental procedure lasted 33 days. For the first 14-day experimental period the group '1' was fed a diet based on 6 kg of hay (mixed grass species, 1<sup>st</sup> cutting) and 6 kg of whole oats grains (both on a fresh basis) (Table 1) divided into two equal daily portions without any additives (C – without feed additives), while the group '2' was fed with the same basal diet but with the addition of the exogenous enzyme preparation (ES – with enzyme addition). After 14 days (the first stage of the experiment) and an additional 5 days for the GIT cleansing from the experimental feed, the treatments were reversed. The group '1' received diet with enzyme supplementation (ES) and group '2' was fed without any additives (C) for another 14 days.

**Table 1.** The analysed nutritive value of feed materials used in the experimental diets, %

Indices	Ingredient	
	hay (mixed grass species)	oat grain
Dry matter	90.9	89.6
Crude protein	12.9	13.1
ADF	36.2	23.6
NDF	59.8	45.8
Starch	4.38	36.9

ADF – acid detergent fibre; NDF – neutral detergent fibre

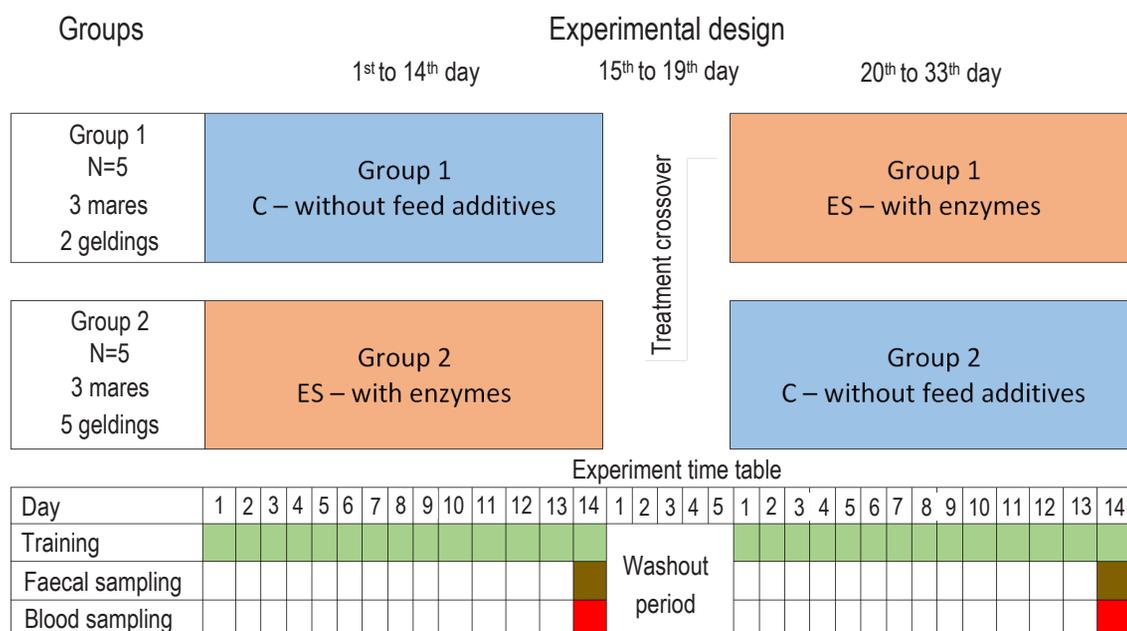


Figure 1. Diagram of the experimental design used in the animal trial

The enzyme product in the amount of 10 ml was sprayed on 100 g of oat middlings, which replaced 100 g of whole oats, and fed as such, per horse, per day. The enzyme product contained a xylanase with activity of 350 000 BXU/g and cellulase – 10 000 ECU/g and which were produced by *Trichoderma reesei*. One BXU is defined as the amount of enzyme that will release 0.06 micromoles of reducing sugars (xylose equivalents) from birch xylan per minute at pH 5.3 and 50 °C. One ECU is the amount of enzyme that will release 0.06 micromole of reducing sugars glucose from hydroxyethyl cellulase per minute at pH 4.8 and 50 °C. Blood and faeces collections were performed at the end of each 14-day long experimental period.

During each experimental period the horses were subject to precisely the same training regime for six days a week. Throughout the study they were not turned out and not exercised at all except as described below. Every horse performed the same work in a given training day. In every week two days were focused on jumping, two on basic dressage, one on hacking and one on special training with continuous gallop. On the day of special training each horse had a blood sample taken before and immediately after training. The blood sampling was performed on day 14 and 33. The special training lasted for 29–31 min and consisted of the following stages: walk – 8 min, trot – 1 km/4 min, gallop – 4 km/8–9 min (speed 450–500 m/min), trot – 1 km/4 min and walk – 5–6 min.

For the digestibility analyses 0.2% of titanium dioxide was mixed with the oat middlings four days before collection on day 10. To obtain representative samples, total collection of the faeces was performed on day 14 for 24 h on an individual horse basis. Titanium dioxide was determined, and samples were prepared according to the procedures described by Kienzle et al. (1997). The digestibility calculations were performed as described in detail in our previous paper (Józefiak et al., 2011) with the use of the following equation (Salem et al., 2015):

$$\text{apparent nutrient digestibility} = 100 - \left( 100 \times \frac{\% \text{TiO}_2 \text{ in feed} \times \% \text{nutrient in faeces}}{\% \text{TiO}_2 \text{ in feed} \times \% \text{nutrient in feed}} \right).$$

The blood was collected by puncture of the external jugular vein using tubes containing EDTA for morphology analysis (Haematology Potassium EDTA Tubes, cat. no. 26.358; Sarstedt, Nümbrecht, Germany) or into tubes from polypropylene for determination of blood parameters (Serum clotting activator Tubes, cat. No. 26.367; Sarstedt, Nümbrecht, Germany). Collections were made before and after the training and between 12:00 and 16:00. Serum was obtained by centrifugation (Mikro 220R, Hettich, Tuttlingen, Germany) at 1000 g at 8 °C for 10 min and stored at –20 °C until analysis. Hormone levels were measured in the serum using the following radioimmunological

assay kits. Obestatin level was determined using a kit (Obestatin RIA Kit, cat. no. RK031-90) purchased from Phoenix Pharmaceuticals, Inc. (Burlingame, CA, USA). Kits for other hormones were purchased from Merck Millipore (Billerica, MA, USA) as follows: adiponectin (Adiponectin RIA Kit, cat no. MADP-60HK), insulin (Insulin RIA Kit, cat no. RI-13K), glucagon (Glucagon RIA Kit, cat no. GL-32K), leptin (Multi-Species Leptin RIA Kit, cat no. XL-85K), ghrelin (Ghrelin Total RIA Kit, cat no. GHRT-89HK and Ghrelin Active RIA Kit, cat no. GHRA-88HK). Metabolic parameters measured in blood serum using enzymatic, colorimetric kits (Pointe Scientific, Inc., Canton, MI, USA) were: serum triglycerides (Triglycerides – GPO Reagent Set, cat. no. T7531), total cholesterol (Cholesterol Reagent Set, cat. no. C7509), high density lipoprotein cholesterol (Liquid auto HDL Cholesterol Reagent Set, cat. no. H7545), protein (Total Protein Reagent Set, cat. no. T7528) as well as glucose (Glucose Oxidase Reagent Set, cat. no. G7519) and lactic acid (Lactate Liquid Reagent Set, cat. no. L7596). For the determination of serum non-esterified fatty acids (NEFA), the diagnostic kit of Wako Chemicals, Inc. (Richmond, VA, USA) was used (cat no. HR Series NEFA – HR 2, 999-34691, 991-34891, 993-351). Kits for measuring enzyme activities were purchased from Pointe Scientific, Inc. (Canton, MI, USA; Alanine aminotransferase/ALT Reagent Set, cat. no. A7525; Aspartate aminotransferase/AST Reagent Set, cat. no. A7560; Liquid  $\gamma$ -glutamyl transferase/GGTP Reagent Set, cat. no. G7571; Alkaline Phosphatase/ALP Reagent Set, cat. no. A7505; Lactate Dehydrogenase/LDH-H Reagent Set, cat. no. L7572; Creatine Kinase/CK Reagent Set, cat. no. C7522).

Faecal particle size distributions were determined using a modified three-sieve method as described in detail by Nørgaard et al. (2004). The sieves consist of 3 segments of different screen meshes (top 4.76 mm, middle 2.4 mm and bottom 1.6 mm). Faeces (500 g/horse) were placed on the top segment with the largest mesh and the faeces were washed through the apparatus by water under pressure until the water flowing out through the bottom sieve was clean.

### Statistical analysis

Statistical analysis of the blood sampling results was performed using the GLM procedure of SAS Software ver. 9.3 (2012; SAS Institute Inc., Cary, NC, USA). All blood parameters were analysed by 2-factorial design, according to the following general model:

$$Y_{ij} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \delta_{ij}$$

where:  $Y_{ij}$  – the observed dependent variable,  $\mu$  – the overall mean,  $\alpha_i$  – the effect of training,  $\beta_j$  – the effect of enzyme,  $(\alpha\beta)_{ij}$  – the interaction between training and enzyme supplementation, and  $\delta_{ij}$  – random error. In cases where the effects were judged significant ( $P < 0.05$ ), means were compared pairwise. Results are given as the means for main effects and root mean square error. For analysis of the results of faecal particle size distribution and digestibility Kolomogorov-Smirnov test was performed for distribution testing and t-Student test for means comparison. In all cases significance level was assumed as  $P \leq 0.05$ .

## Results

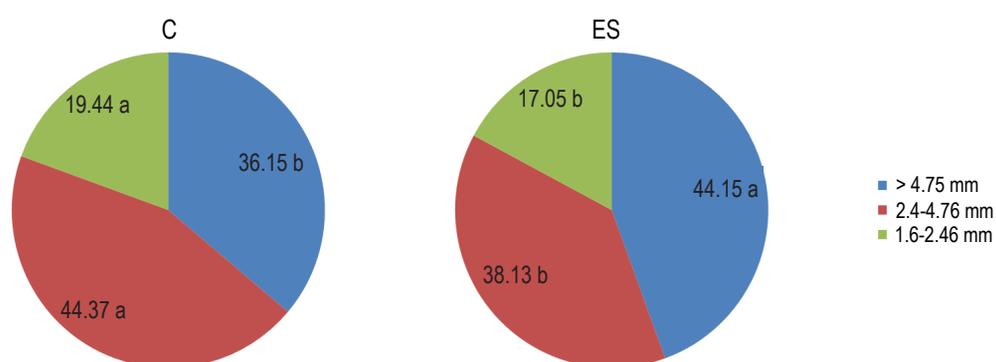
There were no differences in the body weight of the horses between the ES and C treatments at any time. The sieve analysis (Figure 2, 3 and 4) of the faeces from the ES horses indicated less large particles compared with those fed treatment C ( $P < 0.001$ ). In absolute terms there were 8% less large particles in the faeces of the ES horses compared with the C one, which means that there were 18% less large particles in the ES horses compared with the C one on a relative basis. In parallel, in ES horses the presence of medium particles in the faeces was increased ( $P < 0.001$ ). Coupled with this, the digestibility of NDF was markedly higher (12% on a relative basis) in the ES horses ( $P = 0.021$ ) compared with the control one (Table 2). Although starch digestibility was also elevated (by 10% on a relative basis), this effect was not significant ( $P = 0.349$ ). The only parameter of faecal digestibility which was not altered by enzyme supplementation was total tract digestibility of acid detergent fibre.

**Table 2.** The effect of enzyme on faecal digestibility of carbohydrates fractions in horses, %

Indices	Treatments		P-value
	C	ES	
ADF <sup>1</sup>	60.5	60.6	0.984
NDF <sup>1</sup>	60.3 <sup>b</sup>	67.9 <sup>a</sup>	0.021
Starch <sup>1</sup>	73.5	80.7	0.349

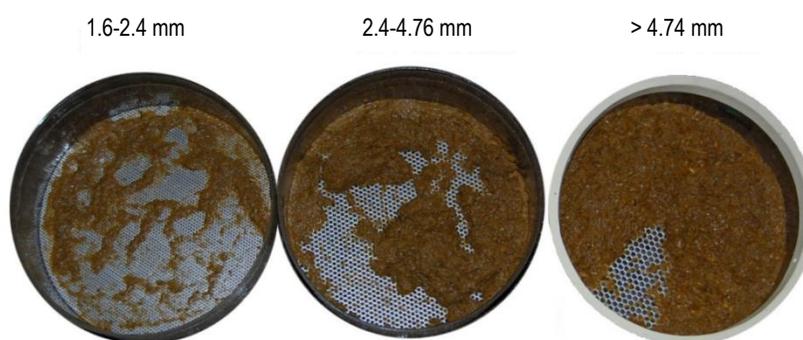
ADF – acid detergent fibre; NDF – neutral detergent fibre; <sup>a,b</sup> – means with different superscripts within each row differ significantly ( $P < 0.05$ ); means represent 5 horses in cross-over design (n = 10)

Enzyme supplementation did not affect blood composition (Table 3) or the majority of examined biochemical blood indices (Table 4) despite having such a marked effect on diet digestibility.



**Figure 2.** Faecal particle size distribution, %

C – control; ES – enzyme supplementation;  $P < 0.001$  for  $>4.75$  mm;  $P < 0.001$  for 2.4–4.76 mm;  $P = 0.068$  for 1.6–2.4 mm; a-b – means with different superscripts for each particle size separately differ significantly ( $P < 0.05$ )



**Figure 3.** Faecal sieves – distribution of the particles without enzyme supplementation (method according to Nørgaard et al. (2004))



**Figure 4.** Faecal sieves – distribution of the particles with the enzyme supplementation (method according to Nørgaard et al. (2004))

**Table 3.** The effect of the enzymes (+/-) and training on blood morphology in horses

Indices	Main effects <sup>1</sup>				Model RMSE <sup>3</sup>	Significance ( $P$ -value)		
	enzyme		training			effect of treatments		interaction
	-	+	-	+		enzyme	training	enzyme × training
Erythrocytes, mmol/l	8.78	8.44	7.69 <sup>b</sup>	9.53 <sup>a</sup>	0.47	0.172	<0.001	0.100
Leukocytes, g/l	8.67	8.72	7.96 <sup>b</sup>	9.44 <sup>a</sup>	1.92	0.853	0.002	0.918
Haematocrit, %	39.6	40.2	35.8 <sup>b</sup>	43.9 <sup>a</sup>	6.16	0.130	<0.001	0.440
Haemoglobin, mmol/l	140	135	122 <sup>b</sup>	153 <sup>a</sup>	132.8	0.286	<0.001	0.683

<sup>1</sup> with (+) or without (-) additive of fibrolytic enzymes in the diet or training; <sup>2</sup> RMSE – root-mean-square error; <sup>a-b</sup> – means with different superscripts within each row for each main effect separately differ significantly ( $P < 0.05$ ); means represent 5 horses in cross-over design

**Table 4.** The effect of the enzymes (+/-) and training on blood biochemistry in horses

Indices	Main effects <sup>1</sup>				Model RMSE <sup>3</sup>	Significance ( <i>P</i> -value)		
	enzyme		training			effect of treatments		interaction
	-	+	-	+		enzyme	training	enzyme × training
Protein, g/dl	6.67	6.52	6.48 <sup>b</sup>	6.71 <sup>a</sup>	0.06	0.118	0.012	0.319
FFA, mg/dl	0.805	0.79	0.73 <sup>b</sup>	0.87 <sup>a</sup>	0.002	0.543	<.0001	0.726
Urea, mmol/l	4.59 <sup>a</sup>	4.04 <sup>b</sup>	4.57	4.06	0.51	0.034	0.058	0.661
Glucose, mg/dl	68.1	69.3	79.0 <sup>a</sup>	58.4 <sup>b</sup>	122.4	0.880	<.0001	0.491
Lactic acid, mmol/g	2.17	2.11	1.90 <sup>b</sup>	2.38 <sup>a</sup>	0.03	0.854	0.028	0.667
AST, IU/l	120	120	117	124	201.1	0.886	0.181	0.541
ALT, IU/l	16.1	16.7	16.0	16.8	11.9	0.664	0.465	0.534
GGTP, IU/l	11.2	11.1	10.5	11.7	4.9	0.954	0.150	0.675
CK, mmol/l	63.3	64.1	63.3	64.1	367.0	0.897	0.911	0.668
LDH-H, IU/l	82.2	89.7	83.3	88.6	357.7	0.269	0.443	0.912
Creatinine, mg/dl	2.82	2.65	2.68	2.79	0.18	0.319	0.507	0.633
Cholesterol, mg/dl	71.7 <sup>a</sup>	66.0 <sup>b</sup>	68.1	69.6	49.4	0.034	0.565	0.554
TG, mg/dl	52.6	47.3	47.5	52.5	63.3	0.082	0.088	0.786
ALP, IU/l	60.5	68.0	62.9	65.6	145.0	0.117	0.455	0.411

FFA – blood insulin-glucagon-glucose; AST – aspartate aminotransferase; ALT – alanine aminotransferase; GGTP – liquid  $\gamma$ -glutamyl transferase; CK – creatine kinase; LDH-H – lactate dehydrogenase; TG – triglycerides; ALP – alkaline phosphatase; <sup>1</sup> with (+) or without (-) additive of fibrolitic enzymes in the diet or training; <sup>2</sup> RMSE – root-mean-square error; <sup>a-b</sup> – means with different superscripts within each row for each main effect separately differ significantly ( $P < 0.05$ ); means represent 5 horses in cross-over design

**Table 5.** The effect of the enzymes (+/-) and training on plasma hormone levels in horses

Indices	Main effects <sup>1</sup>				Model RMSE <sup>2</sup>	Significance ( <i>P</i> -value)		
	enzyme		training			effect of treatments		interaction
	-	+	-	+		enzyme	training	enzyme × training
Adiponectin, ng/ml	4.24	4.30	3.82 <sup>b</sup>	4.72 <sup>a</sup>	1.21	0.898	0.019	0.548
Insulin, ng/ml	0.59	0.64	0.93 <sup>a</sup>	0.30 <sup>b</sup>	0.07	0.927	<.0001	0.135
Glucagon, pg/ml	183	186	172	196	1518	0.868	0.093	0.925
Leptin, ng/ml	1.82 <sup>b</sup>	2.96 <sup>a</sup>	2.37	2.41	0.76	0.008	0.923	0.169
Obestatin, pg/ml	168 <sup>a</sup>	129 <sup>b</sup>	143	154	963	0.002	0.338	0.416
Ghrelin active, pg/ml	10.3	10.5	13.5 <sup>a</sup>	7.35 <sup>b</sup>	28.2	0.933	0.008	0.070
Ghrelin total, pg/ml	982	883	837	1028	73987	0.341	0.072	0.005

<sup>1</sup> with (+) or without (-) additive of fibrolitic enzymes in the diet or training; <sup>2</sup> RMSE – root-mean-square error; <sup>a-b</sup> – means with different superscripts within each row for each main effect separately differ significantly ( $P < 0.05$ ); means represent 5 horses in cross-over design

Nevertheless, statistically significant ( $P < 0.05$ ) reductions in blood cholesterol and urea concentrations were stated in the ES horses. The ES treatment also tended to reduce blood triglyceride concentration; however, this effect was not statistically significant ( $P = 0.082$ ). The ES treatment resulted also in significant and large quantitative changes in the levels of hormones (Table 5) involved in feed behaviour and energy homeostasis, i.e., leptin and obestatin. Leptin concentration was increased and obestatin one decreased. No changes were detected for any of the other measured hormones, i.e., insulin, glucagon, adiponectin and ghrelin.

In addition to investigating the effect of enzyme supplementation, the study allowed to collect data

relating to the influence of training on the metabolism of the horse. In general, training increased number of erythrocytes, haematocrit and content of haemoglobin ( $P < 0.001$ ) as well as number of leukocytes ( $P = 0.002$ ), but there was no interaction between enzyme addition and training for these blood parameters. Some metabolic indices in the blood were also influenced by physical activity (Table 5). Lactic acid and free fatty acid contents were elevated and glucose depleted with training. Training resulted in a temporary elevation of blood protein and triglyceride content and a reduction in urea concentration; however all of these changes were only trends from statistical point of view ( $P = 0.10-0.05$ ). Physical effort evoked a large

impact on the main factors responsible for anabolic/catabolic pathways and turnover of carbohydrates, lipids and proteins, including a reduction in insulin concentration ( $P < 0.001$ ) accompanied by a numerical but not statistically significant ( $P = 0.093$ ) elevation of glucagon level. As a result, the mean insulin/glucagon ratio (pg/pg; calculated from the data presented in Table 4) was decidedly reduced after training (from 4.85 to 1.80 for C group; from 5.95 to 1.26 for ES group). Also, important changes were observed for concentration of adiponectin (increased;  $P = 0.019$ ) and active ghrelin (decreased;  $P < 0.008$ ). The training regime did not influence concentrations of the other investigated hormones, i.e., leptin, obestatin and total ghrelin.

## Discussion

Hay is a commonly used ingredient for feeding horses. However, the energy requirement of modern sport horses has increased significantly over time, and with hard work may be 90% greater than their maintenance requirement according to National Research Council (NRC, 2007). Horses usually consume about 2.5% of their body weight per day, in dry matter (DM), and cannot physically consume enough feed to meet this increased energy demand, thus high-energy cereal grains and fats are included in the diet. This may be in conflict with equine digestive physiology and lead to disorders such as caecal acidosis, colic and laminitis that are caused by increased fermentation in the hindgut. Carbohydrate metabolism is directly inter-linked with the status of circulating hormones (e.g. insulin) and the development of insulin resistance in horses (Kienzle et al., 1997). Diets providing an excess of easily digestible sugars, such as those released from starch, can have a significant effect on the development of the metabolic diseases, including obesity and metabolic syndrome. Thus, the right composition and digestibility of the ration are crucial to the health and performance of horses. This is especially important for sport horses, for which a high-digestive diet is desirable, but which presents the risk of overfeeding that can significantly impair the performance.

**Carbohydrate digestibility and sieve faecal analysis.** In the present experiment, the potential value of fibrolytic enzymes in traditional fibrous rations based on hay and whole oats has been examined. Carbohydrase preparation has previously been shown to decrease the DM digestibility of diet based on an alfalfa hay and had no effect on

the DM digestibility of whole oat grains (Hainze et al., 2003). The present work in this respect is partly consistent as it suggests that acid detergent fibre (ADF) digestibility coefficients have not been improved by enzyme supplementation, but the neutral detergent fibre (NDF) digestibility has clearly increased. Obtained digestibility data are in agreement with the sieve analyses of the faeces. The clearly visible increase in NDF digestibility as a result of the use of ES was reflected in the reduction in the number of large particles and the increase in the number of medium particles in the faeces. It has been suggested that such finding requires more attention and may be a suitable method to predict fibre digestibility *in vivo*, as this method can easily be applied in practice. However, in the case of fibrolytic enzymes application and their effects on nutrient digestibility in horses there are still scarce data based on *in vivo* experiments. An experiment carried by Salem et al. (2015) showed that the use of xylanase, cellulase and their combination increased feed intake as well as digestibility of DM, organic matter, crude protein, ADF and NDF from wheat bran and oat straw based diet. The opposite results were obtained by O'Connor-Robinson et al., 2007 using cellulase. They observed numerical decrease in digested DM, NDF and nitrogen and a significant worsening of ADF digestibility and decrease in digestible energy content as the effects of using cellulase. However the exact reason of the decrease in digestibility was not explained (O'Connor-Robinson et al., 2007). It is suggested that the source of plant matter in horse diet as well as its treatment and preparation are important factors affecting digestibility. In addition, no dose response manner was observed when using enzymes in horses (Murray et al., 2007). Moreover, one of the explanations used in the event of negative or no enzyme activity in horses may be microbial/ exogenous enzyme interactions that can lead to the reduction in fibre digestibility (Murray et al., 2007), which may have correlation with their antimicrobial effect observed by Salem et al. (2015) in *in vivo* study. On the other hand, *in vitro* studies have shown a positive effect of enzymes on both fibre digestibility as well as cellulolytic bacteria populations (Mohammadabadi et al., 2018). All of the above-mentioned results show a large variability of the effects of using exogenous enzymes, which suggests that increased efforts should be undertaken in the case of enzyme use in horse nutrition in terms of their mode and place of action in the intestines of horses, as well as enzyme-microbiota-host relationship.

**Blood biochemistry and hormonal response.**

In the study we attempted to measure possible effects of the applied enzymes and training on blood morphology and biochemistry and hormonal response. According to our knowledge, it is the first attempt to characterize blood parameters in terms of fibrolitic enzyme used in training horses. As expected, training had a significant effect on all measured morphology parameters, while enzyme treatment did not induce any marked changes. Taking into consideration all examined blood biochemistry parameters, ES treatment caused a reduction of urea concentration regardless of a training regimen. This might be linked with better feed utilization and a lower rate of deamination and perhaps even improved nitrogen retention. Alternatively, the enzyme addition may cause more carbohydrate available for fermentation, which enables the hindgut bacteria to utilize more N and hence less is voided. Although not of much relevance to the performance of the racehorses, but potentially of benefit to their health as long-living animals, was the effect of the exogenous enzyme on reducing blood cholesterol ( $P < 0.034$ ). In the available literature, the information on the effects of nutrition on the cholesterol levels in horses is scarce. It was earlier observed that it can be lowered by regular training (Dunstan et al., 2019); however, in the present study, only the effect of enzyme treatment was visible on cholesterol level. In the case of nutritional factors, bioactive plant extracts, i.e., garlic extract due to the presence of allicin, have been mentioned as anti-cholesterol agents (Elghandour et al., 2018). However cholesterol level is also considered as closely correlated with dietary fat as well as exercise-induced glucose changes in blood plasma (Hambleton et al., 1980), but such a trend in the correlation of the examined parameters was not observed in the current study.

In the present trial even though NDF utilization increased after enzyme supplementation, increased glucose and insulin levels were not observed, presumably as a result of a gradual rather than acute release of glucose from the fodder and/or NDF being fermented. Thus the additional energy would be absorbed as free fatty acids rather than presented as an overflow of free sugars to the horse. There was no change in insulin concentration with enzyme supplementation ( $P > 0.05$ ) and the observed levels of insulin remained in the normal reference range (Cartmill et al., 2005). ES treatment was responsible for elevating blood leptin and reducing obestatin concentration without exerting an effect on the

ghrelin level. Leptin, ghrelin and obestatin are treated as hormones responsible for appetite control and whole-body energy balance, playing opposite roles. Although it is considered that obestatin, leptin and ghrelin are key factors involved in the energy balance in humans and animals (Beasley et al., 2009, Wójcik-Gładysz and Szlis, 2016), the exact roles and possible interactions of these hormones in horse nutrition are unclear. Thus it is difficult to unequivocally interpret the changes noted after enzyme treatment, but we presume that the elevation of leptin content with ES in the absence of changes in ghrelin level may suggest tendency of the organism to reduce feed intake without delaying gastric emptying (i.e., lower level of obestatin). Regardless of the equivocal role of obestatin in control of appetite, studies show that it suppresses gastric emptying and jejunal contraction in the rat (Zhang et al., 2005). In this context, the reduction of obestatin following ES treatment may have a positive aspect for sport horses as it would facilitate improved food passage through the digestive tract. However, the increased leptin and decreased obestatin blood levels after fibrolitic enzyme treatment can be a sign of better fodder efficiency. According to some researchers, the ratio of ghrelin to obestatin is also important for the regulation of energy balance (Guo et al., 2008). The ghrelin to obestatin ratio is reduced in a number of gastrointestinal diseases such as inflammatory bowel disease and chronic atrophic gastritis (Seim et al., 2011). In ES horses, the ratio of active ghrelin to obestatin was elevated in comparison to control animals, which may thus be considered to be beneficial. The enzyme treatment resulted in increased blood leptin concentrations, which may be considered as a negative effect since leptin is positively correlated with body conditioning score (Buff et al., 2002; Frank et al., 2006) and high leptin levels are correlated with laminitis (Carter et al., 2009). However, in the present trial, the leptin levels were very low and even in the ES group it did not exceed 3.23 ng/ml while in other studies much higher values (about 5 ng/ml) were determined and still considered as low, without any relation to digestive disorders and development of laminitis (Kienzle et al., 1997).

The present study indicates that enhanced digestibility of the structural carbohydrates in particular NDF, enables horses to extract more energy from high fibre diets when appropriate fibrolitic enzymes are used. With such treatments, the release of energy from the diet is controlled and should not precipitate any negative effects such as obesity and laminitis. The additional energy arising

from the diet as a result of ES treatment is due to greater fermentation and likely production of volatile fatty acids (VFA), which clearly did not disturb the metabolism or hormonal profile of the horses in such a way that there would be concerns regarding metabolic disorders. Such an effect, when coupled with the right training regime, may be ideal for the development of sport horses. As expected the number of erythrocytes, concentration haemoglobin and the haematocrit value were elevated in groups of animals undergoing training. Such changes are typical for horses and results from the mobilisation of the splenic blood-cell reservoir caused to a large extent by release of the catecholamines (Persson, 1967). The higher levels of catecholamines after exercise may also be responsible for the simultaneous reduction in insulin level (McKeever, 2002). In this study, the higher lactate and free fatty acids, and lower glucose levels were noted after training, and the extent to which this happens may be dependent on the type of exercise. In the present study, the mix of aerobic and anaerobic conditions led to the recruitment of both oxidative (type I and/or IIA) and glycolytic (type IIB) muscle fibres (Voss et al., 2002), which would explain the obtained results. Only three hormones were statistically affected by exercise; the insulin and ghrelin (active) levels diminished while the adiponectin content increased. The influence of training on adiponectin concentrations was not uniform. No changes in the level of this hormone were noted in standardbred horses exposed to treadmill exercise (Gordon et al., 2007). A similar response was observed in cycling humans (Ferguson et al., 2004). However, an increase in plasma adiponectin concentration after moderate walk/jog training was also reported (Kriketos et al., 2004). It is possible that changes in adiponectin level might depend on the intensity and duration of exercise (Kriketos et al., 2004). Taking into consideration the level of insulin and adiponectin noticed in the present experiment it is possible that there was an improvement in insulin sensitivity in horses after such type of exercise applied. However, the diet did not influence the response of animals to training. Ghrelin concentrations fluctuated with diet and exercise and were independent from those of insulin and adiponectin. The important and stimulatory role of ghrelin in the regulation of feed intake during rest is obvious (Sobrinho Crespo et al., 2014). However, it is more difficult to comment on the effect of changes in this hormone as a result of physical effort. Our investigations indicate diminished post-training concentrations of active

ghrelin with a lack of ghrelin change in enzyme supplemented horses. From a physiological point of view, the reduction of the active ghrelin is easy to explain by the fact that during physical training, feed intake (stimulated by ghrelin) is not a priority, and the hormonal milieu would be more focused on increased energy expenditure. Previous data show no change in active ghrelin content during exercise in horses (Gordon et al., 2007). The concentration of this form of hormone does not alter in mares and stallions during intense activity (Bis-Wencel et al., 2013). In the case of the above-mentioned study on the usage of xylanase, cellulase and their combination, the blood parameters that increased by enzyme treatment were ALT and AST activity and total protein content (Salem et al., 2015). Comparing with the results of the present study the enzyme supplementation did not affect these blood parameters. The only significant effect was recorded in terms of total protein content, which was increased by training. In conclusion, the results obtained after exercise did not differ significantly between horses fed diet with or without enzyme treatment. Statistical analysis showed no interaction between training and fibrolytic enzyme addition for any investigated blood parameters, thus further studies are advised in terms of the effects of exogenous enzyme usage on horse physiology.

## Conclusions

The study showed that supplementation of a horse diet with fibrolytic enzymes seems to improve feed digestibility which may influence the energy balance; however, due to divergent results – both positive and negative obtained in various studies, enzyme preparations for different groups of horses should be carefully selected. The obtained results indicated also that the ability of horses to exercise remained unchanged when the fibrolytic enzymes are added to the diet, thus well-designed and used enzyme preparations may be beneficial for sport and recreation horses.

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