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Starch in horse diet improves feces microbiota, *in vitro* digestibility of fiber and dry matter

Škrob u obroku konja poboljšat će mikrobiotu fecesa, *in vitro* probavljivost vlakana i suhe tvari

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Abstract

Higher concentrations of starch in horse diet may shift starch degradation from small intestine further to hindgut and cause undesired alternation of the hindgut microbiota leading to unsatisfied fiber fermentation. The study evaluated the effect of the daily starch intake levels of 0.3, 0.6 and 0.9 g/kg body weight (BW) of the animal on the fecal *in vitro* digestibility of neutral detergent fiber (IVNDFD) and dry matter (IVDMD) and whether changes in fecal amylolytic bacteria, cellulolytic bacteria and lactobacilli number occur. Three horses were fed in three periods with three starch levels in the trial, designed as 3x3 latin square. The main influences were the level of starch in the horse diet and the effect of an individual animal on *in vitro* digestibility of three forages (straw, clover and meadow hay). The results showed that with increasing daily starch intake the significant increase in average values of IVNDFD 9.46 − 11.49% and IVDMD 3.42 − 8.09% depending the forage type were observed. The individual animal showed an influence on tested digestibility parameters (all $P<0.001$) except the IVDMD for meadow hay ($P=0.126$). When the intake starch level was 0.9 g/kg BW in compare to 0.3 g/kg BW, the tenfold higher number of lactobacilli, amylolytic and cellulolytic bacteria in the feces was recorded. It can be concluded that the daily level of starch in the horse diet up to 0.9 g/kg BW has a positive effect on fiber digestibility and feces microbiota.

Keywords: feces microbiota, *in vitro* fiber digestibility, starch in horse diet

Sažetak

U slučaju viših koncentracija škroba u hrani konja mogući je pomak razgradnje škroba iz tankog crijeva i njegova povećana fermentacija u debelom i slijepom crijevu čime se narušava mikrobiota u crijevima konja i fermentacija vlakana. Istraživanje je ispitalo utjecaj dnevne razine škroba od 0,3, 0,6 i 0,9 g/kg tjelesne mase (TM) životinje u obroku konja na fekalnu *in vitro* probavljivost neutralnih detergent vlakana (IVNDFD) i suhe tvari (IVDMD), te da li dolazi do promjena u broju amilolitičkih, celulolitičkih bakterija i laktobacila u fecesu. Za istraživanje, dizajnirano kao 3x3 latinski kvadrat, korištena su tri konja koja su hranjena u tri perioda s tri različite dnevne razine škroba. Glavni ispitani utjecaji bili su razina škroba u obroku konja i utjecaj pojedine životinje na *in vitro* probavljivost tri voluminozna krmiva (slama, djetelina i livadno sijeno). Rezultati su pokazali da je s porastom dnevne razine škroba proporcijonalno zabilježeno i značajno povećanje prosječnih vrijednosti IVNDFD 9,46 – 11,49% te IVDMD 3,42 – 8,09% ovisno o tipu voluminoze. Pojedina životinja imala je signifikantan utjecaj na testirane parametre probavljivosti (svi $P<0,001$) osim kod IVDMD za livadno sijeno ($P=0,126$). Kada je udio škroba u obroku bio 0,9 g/kg TM u odnosu na 0,3 g/kg TM, zabilježena je deset puta veća brojnost laktobacila, amilolitičkih i celulolitičkih bakterija u fecesu konja. Može se zaključiti da dnevna razina škroba u obroku konja do 0,9 g/kg TM ima pozitivan utjecaj na razgradnju vlakana i mikrobiotu fecesa.

Ključne riječi: *in vitro* probavljivost vlakana, mikrobiota fecesa, škrob u obroku konja

Introduction

Horses are grazing animals that in wild mainly spend their time grazing grass. Horse domestication and confinement to stables with little available pasture shifted horse feeding management from continuous grazing to few daily rations, comprising of low energy hay supplemented with high energy concentrate rich in starch. Hay is the main source of fiber and consequently the most common type of forage fed to horses. Fiber is the most important ingredient in every horse diet and it comprises more than 50% of the diets; fiber provides all the energy horses need for everyday maintenance metabolism, proper digestive system function, the ability to move food particles efficiently through the gut and the ability to conserve water and electrolytes (Briggs, 2007).

High starch diet has some shortcomings. It induces an alteration in gastrointestinal motility and large fluid shifts, has an influence on gut pH and microflora and can depress fiber fermentation (Clarke et al., 1990; De Fombelle et al., 2003; Lawrence, 2005; Willing et al., 2009). Fiber is fermented in the cecum and colon, horse hindgut, and starch in its optimal amounts is digested in the small intestine (Hintz and Cymbaluk, 1994). When horses are fed with higher amounts of starch, some starch can escape small intestine digestion and is fermented in hindgut thus lowering its energetic value and decreasing luminal content pH and may depress fiber digestibility (Lawrence, 2005) as fibrolytic bacteria are predominantly low pH intolerant bacteria (Shirazi-Beechey, 2008). Potter et al. (1992) suggested that maximal amount of starch, based on oat (*Avena sativa*) usage, in horse diet should

not exceed 3.5 to 4 g/kg body weight (BW). Cuddeford (1999) however, in his overview of other starch digestibility studies suggested much lower amounts; 2 g/kg BW or lower depending on the starch grain source. The amount of starch that can be tolerated varies with the type of grain and the type of grain processing (Lawrence, 2005).

Starch that escapes small intestine digestion and is fermented in the hindgut can lead to increased numbers of amylolytic bacteria, including streptococci (*Streptococcus* spp.) and lactobacilli (*Lactobacillus* spp.), decrease pH and decrease the number of cellulolytic bacteria (Medina et al. 2002; De Fombelle et al., 2003; Harlow et al., 2016). It has been suggested that changes in intestinal microbiota induced by starch usage elevates the risk for digestive problems in horses (Shirazi-Beechey, 2008).

Based on aforementioned the hypothesis in this study was that with the starch level increase in the horse diet the reduction in feces cellulolytic bacteria number and in neutral detergent fiber digestibility will be observed. To test this hypothesis in the presented study was examined the effect of daily starch level at 0.3, 0.6 and 0.9 g/kg BW of the animal on:

i) fecal *in vitro* digestibility of neutral detergent fiber (IVNDFD),
ii) fecal *in vitro* digestibility of dry matter (IVDMD), and
iii) whether changes in fecal amylolytic bacteria, cellulolytic bacteria and lactobacilli occur with increasing starch level.

Materials and methods

The trial was arranged as the latin square (3x3) where three horses were fed in three 13 days periods with horse feed containing the total daily starch level of 0.3, 0.6 and 0.9 g/kg BW of the animal. Animal handling was in accordance with European Parliament and Council Directive 2010/63/EU on the protection of animals used for scientific purposes.

Horse body condition, feeding and feces sample preparation

Three horses used for trial were selected on the basis of their physiological characteristics i.e. horses of the similar age and similar BW (Table 1). Equation defined by Carroll and Huntington (1988) was applied in BW determination. Prediction of BW using body measurements is exact (R^2=0.93 − 0.91), practical and easy to take and is usually done in horse experiments when no scale possibility (Bene et al., 2014). Daily routines of horses were similar, 60 min/day exercise equally divided on walking, trotting and galloping; horse number 1 showed some lower levels of activity during exercise. At rest, horses were kept in the same microclimate housing conditions in individual stalls of 12 m^2.

Horses were fed with the same hay during the whole trial period; feed ration 12 kg/day of meadow hay (Table 2) was divided proportionally into three parts (feeding at 7:00, 13:00 and 19:00 hours). Three feeding treatments differed in the daily proportion of starch in horse diets (0.3, 0.6 and 0.9 g/kg BW). Source of starch in the

diet was pelleted concentrate for horses Centaur Basic (Kušić promet d.o.o., Croatia) with starch content 300 g/kg DM.

Before feces collection, every horse was on ten days adaptation period. After adaptation to treatment three following days' feces was collected rectally, plastically sealed, placed in an airtight bag and stored anaerobically at 4℃. At the end of feces collection, daily feces were combined and thoroughly mixed. One part of each combined fecal sample was used for microbiological analysis and pH determination (IQ150, IQ Scientific, USA). The other part of feces was used for determination of IVDMD and IVNDFD.

Table 1. Horse characteristics

Horse	Age (years)	Sex	BW (kg)	Heart girth (m)	Body length (m)
1	13	Mare	574.2	1.98	1.74
2	12	Gelding	572.4	2	1.7
3	12	Gelding	619.1	2.05	1.75

BW: body weight

Microbiological analysis of feces

To determine the total number of cellulolytic, lactobacilli and amylolytic bacteria in grams, a modified protocol was used according to Bairagi et al. (2002): on 20 g of fresh feces was added 180 mL of Buffer Peptone Water (Merck, Germany) with Tween 80 (Merck, Germany) and homogenized in stomacher bag (Bag Mixer, Interscience, France) for 3 min. Initial dilution was used for ten serial 1:10 dilutions. From each dilution in duplicate, 0.1 mL aliquots were uniformly spread on a nutrient medium surface by L-stick on each nutrition agar plate. For cellulolytic bacteria carboxymethylcellulose nutrition agar (CMC, pH 7) was prepared as follows (g/L): carboxymethylcellulose, 10; KH_2PO_4, 4; Na_2HPO_4, 4; Tryptone, 2; $MgSO_4 \times 7H_2O$, 0.2; $CaCl_2$, 0.001; $FeSO \times 7H_2O$, 0.004; agar 15. For amylolytic bacteria starch nutrition agar (pH 7) was prepared as follows (g/L): starch, 10; KH_2PO_4, 4; Tryptone, 2; $MgSO_4 \times 7H_2O$, 0.2; $CaCl_2$, 0.001; $FeSO_4 \times 7H_2O$, 0.004; agar, 15. Commercial MRS agar (Merck, Germany) was used for lactobacilli. Inoculated plates (CMC and starch agar) were incubated for 24 hours at 37 °C (± 1 °C) under anaerobic conditions while MRS agar plates were incubated for 72 hours at 37 °C (± 1 °C) in semi-anaerobic conditions. After incubation increased bacterial colonies were counted and CFU/g was determined.

Sample preparation for *in vitro* digestibility

Three forages (straw, meadow and clover (*Trifolium sp.*) hay) were divided at half and subject to DM determination (103℃, 24 hours) and preparation of dry sample;

drying at 60℃ (24 hours) and grounding through 1-mm screen (cyclone mill, Cyclotec 1093, Foss Tocator, Hoganas, Sweden) or 2-mm screen (cemotec mill, Cemotec 1090 Sample mill, Foss Tocator, Hoganas, Sweden). 1-mm samples were analyzed for DM (4 g of sample, 103 °C, 4 hours), crude protein and neutral detergent fiber (NDF) according to EN ISO 16472:2006 protocol on a Fibretec FibreCap 2021/2023 system (FOSS Analytical, Hillerød, Denmak). Crude protein (CP) was determined using the Kjeldahl procedure to assay total N. The total N value was multiplied by 6.25 to obtain the CP. 2-mm samples were used for IVDMD and IVNDFD.

Table 2. Neutral detergent fiber (NDF) and crude protein (CP) in evaluated forage samples

	CP (g/kg DM)	NDF (g/kg DM)
Straw	34.2	834
Meadow hay	60.2	620
Clover hay	103.9	636

Fecal *in vitro* digestibility

Fecal *in vitro* digestibility was performed according to Earing et al. (2010) in Ankom Daisy^{II} incubator (ANKOM Technology, Fairport, NY, USA). 2-mm samples of straw, clover and meadow hay were weight (0.5 g) in six repetitions in acetone-washed F57 bags with a 25 µm pore size (ANKOM Technology), which were then heat-sealed and placed into three Daisy^{II} incubation jars containing incubation solution preheated at a temperature of 39 °C. Each jar represented one animal; in total 18 bags (six repetitions of straw, clover or meadow hay) per jar including two blanks were incubated for 48 hours. Incubation solution comprised a buffer, mineral solution, and feces (Earing et al., 2010). Feces collection was performed as described. For each jar, 0.177 kg of homogenized combined feces was added to 1,600 mL of buffer/mineral solution preheated at a temperature of 39 °C. All solutions were kept under a stream of CO_2 to maintain anaerobic conditions.

At the end of incubation, the bags were washed in iced water to remove bacterial debris and left to dry overnight (60℃). Before undigested NDF determination, the bags were weighed for IVDMD determination. Undigested NDF was quantified by a previously mentioned procedure for NDF determination (EN ISO 16472:2006 protocol), but directly in F57 bags. IVDMD (%) was expressed as a digested fraction of the initial sample. The digested NDF was calculated by subtracting the initial NDF weighed into the bag before incubation and undigested NDF, and the IVNDFD (%) was expressed as a digested NDF fraction of the initial NDF.

Statistical analysis

In vitro digestibility data of three evaluated forages were analyzed as a repeated latin square (in total six repetitions) using the GLM procedure in SAS 9.3 (SAS Institute, Cary, NC, USA). Each latin square was thought of as an independent replication of the entire experiment. Main effects in the model included feeding treatment and animal. Differences and interactions were considered significant when $P<0.05$.

Results and discussion

The data to determine the effect of daily starch concentrations in horse diet up to 0.9 g/kg BW on feces pH (Table 3), feces microbiota (Table 4, Table 5 and Table 6) and on IVNDFD and IVDMD in three forage types (Table 8) are presented. When fiber fermentation in horses is disrupted, health disorders are more frequent. High grain diet, with high starch intake, negatively affects fiber fermentation as fermentation of starch and sugars is connected with the development of acidic conditions (low pH) in cecum and colon (Clarke et al., 1990; Rowe et al., 1994).

The collected feces in this experiment, regardless of the starch intake, were firm and compact with non-observed soft and unformed parts usually connected with low pH (6.2) of feces (Rowe et al., 1994). Feces pH showed a decline with the increase of starch in the diet (Table 3) with the lowest value observed in horse 3 in the diet containing starch 0.9 g/kg BW (pH 6.8).

Table 3. pH value of feces of three horses in three feeding treatments (I: starch 0.3 g/kg BW; II: starch 0.6 g/kg BW; III: starch 0.9 g/kg BW)

	pH		
	Treatment I	Treatment II	Treatment III
Horse 1	8.02	7.25	6.9
Horse 2	7.69	6.98	7.14
Horse 3	7.95	7.29	6.8
Average	7.89	7.17	6.95

Starch hydrolysis results in the production of organic acids, mainly lactic acid that reduces pH (Hinz and Cymbaluk, 1994). Willard et al. (1977) showed that pH in the hindgut is defined by the type of horse diet; the cecal pH decreased from 7.22 to 6.12 six hours after feeding concentrated feed. pH values observed in this study (in average from 7.89 to 6.95 depending on the starch content i.e. treatment) were far from pathologically low pH. A cecal pH value of 6 represents sub-clinical acidosis, and below 6 there is a considered risk in the development of imbalanced fermentation and gastrointestinal illnesses (Radicke et al., 1991). In accordance with

here observed decrease in pH of feces with the increase of starch in horse diet, Zeyner et al. (2004) showed a significant increase in feces pH value (from 6.36 to 6.68) when the gradual increase in hay intake was applied (from 0.5 to 1 kg/100 kg BW × day). Richards et al. (2006) showed that fecal pH value is a good indicator of starch fermentation in the hindgut and of hindgut health and microbiota status.

In this study the number of amylolytic bacteria, cellulolytic bacteria and lactobacilli increased with the increase of starch in the horse diet (Table 4, Table 5 and Table 6). Amylases, the enzymes that degrade starch, are predominantly extracellular enzymes (Vihinen and Mantsiila, 1989) thus releasing products of the starch degradation in the intestinal lumen making it available for intestinal microbiota. In all three treatments was unexpectedly observed that the cellulolytic bacteria were among numerous ones, except in treatment II (0.6 g/kg BW) where amylolytic bacteria had a bit higher number than cellulolytic bacteria (Table 5).

Table 4. Microbiological analysis of feces of three horses in first treatment (starch 0.3 g/kg BW)

	Starch 0.3 g/kg BW		
	Cellulolytic (CFU/g feces)	Amylolytic (CFU/g feces)	Lactobacilli (CFU/g feces)
Horse 1	1×10^7	2×10^6	1×10^7
Horse 2	1.2×10^7	1.7×10^7	1×10^7
Horse 3	1.2×10^7	1.2×10^7	1.3×10^7
Average	1.1×10^7	1×10^7	1.1×10^7

CFU: colony forming units

Changing diet from grass to grain increases population of lactate-producing bacteria that favor starch as substrate and decreases acid intolerant cellulolytic bacteria (Shirazi-Beechey, 2008). As excessive lactic acid is produced pH is decreased (Medina et al., 2002). At pH 6 − 6.1 cellulolytic bacteria are suppressed and completely inhibited at pH < 5.9 (Miwa et al., 1997); cellulolytic bacteria are predominantly acid-intolerant bacteria (Shirazi-Beechey, 2008). Lower enumerations of cellulolytic bacteria in diets with over usage of concentrates are measured (Medina et al. 2002; De Fombelle et al., 2003; Harlow et al., 2016). However, similarly to observation obtained in this study, Moore and Dehority (1993) found 3.55 times more cellulolytic bacteria in the colon when concentrate diet with an average starch intake of 0.53 kg/day was fed to ponies in comparison to forage diet. The concentrate used in the study was based on corn (Moore and Dehority, 1993). In this study negative effect of starch fermentation on cellulolytic bacteria was not observed as inhibitory pH values (pH < 5.9), regardless of starch content in horse diet, were not observed (lowest pH 6.8 was observed in treatment III in horse 3, Table 3). Amylolytic bacteria

increased with the higher starch intake (Table 4, Table 5, Table 6). Primarily substrate for amylolytic bacteria is starch, thus the observed higher number of this type bacteria in diets with more starch is expected (Harlow et al., 2016).

Table 5. Microbiological analysis of feces of three horses in second treatment (starch 0.6 g/kg BW)

	Starch 0.6 g/kg BW		
	Cellulolytic (CFU/g feces)	Amylolytic (CFU/g feces)	Lactobacilli (CFU/g feces)
Horse 1	9.5×10^7	9×10^7	5.5×10^7
Horse 2	8.5×10^7	1×10^8	6×10^7
Horse 3	6×10^7	8.5×10^7	4.5×10^7
Average	8×10^7	9.2×10^7	5.3×10^7

CFU: colony forming units

Table 6. Microbiological analysis of feces of three horses in third treatment (starch 0.9 g/kg BW)

	Starch 0.9 g/kg BW		
	Cellulolytic (CFU/g feces)	Amylolytic (CFU/g feces)	Lactobacilli (CFU/g feces)
Horse 1	6×10^8	3.8×10^8	3.2×10^8
Horse 2	1×10^9	5.7×10^8	2.5×10^8
Horse 3	4×10^8	3.6×10^8	5×10^8
Average	6.7×10^8	4×10^8	3.6×10^8

CFU: colony forming units

In all three evaluated treatments lactobacilli were lowest and their number is in accordance with observed pH change (Table 3); higher starch content in horse diet was accompanied with lower pH value (Table 3) and higher lactobacilli number in feces (Table 4, Table 5 and Table 6). When high starch diets compared to high forage diets are fed to horses a tenfold increase in lactobacilli count on Rogosa agar is detected in feces of horse fed high starch diets (Willing et al., 2009). Medina et al. (2002) showed that high starch diets results with excess lactic acid production in the hindgut (2.43 times higher lactic acid in the cecum and 2.61 times higher lactic acid

in the colon in high starch diets compare to high forage diets) leading to decrease in the pH of the luminal content.

The fecal *in vitro* digestibility significantly differed in all three evaluated forages between horses except for the IVDMD in meadow hay where IVDMD was similar between horses (Table 7). Results indicate that horses differed in their response to higher starch content in diets. Willing et al. (2009) with cluster analysis of microbial diversity in feces of horses fed with high forage and high concentrate showed that samples tended to group according to diet as well as within horse, marking that individual horse effects microbial diversity and stability. In this study, the lowest digestibility values, both for IVDMD and IVNDFD, were measured in horse 1 that showed lower levels of activity during exercise. The horse activity and exercise could explain digestibility divergences observed between horses. Pagan et al. (1998), Bergero et al. (2002) and Goachet et al. (2010) showed that horse exercise influences digestibility; light exercise improves digestibility while heavy work reduces.

Table 7. Fecal *in vitro* neutral detergent fiber digestibility (IVNDFD) and *in vitro* dry matter digestibility (IVDMD) of three evaluated forages (straw, meadow hay and clover hay) – horse influence

	Straw		Meadow hay		Clover hay	
	IVNDFD (%)	IVDMD (%)	IVNDFD (%)	IVDMD (%)	IVNDFD (%)	IVDMD (%)
Horse 1	32.08	25.82	33.73	38.27	39.48	42.61
Horse 2	36.6	27.67	34.37	38.51	40.76	42.07
Horse 3	35.47	26.62	36.3	39.31	43.01	43.38
P value	<0.001	<0.001	<0.001	0.126	<0.001	<0.001

The increase of starch in horse diet up to 0.9 g/kg BW had a positive effect on IVNDFD and IVDMD in all three evaluated forages (Table 8) with the highest digestibility values observed in treatment with the highest starch content (0.9 g/kg BW). High concentrate diet shows higher organic matter digestibility than high fiber diet (Drogoul et al., 2001), starch is readily digestible than fiber (Hintz and Cymbaluk, 1994; Cuddeford, 2001). Dry matter digestibility of high concentrate diet is significantly higher than digestibility of high hay diet (73.08 vs 63.66%; Moore and Dehority, 1993). However, concentrate usage and resulting low intestinal pH can disturb fiber digestibility (Clarke et al., 1990; Hintz and Cymbaluk, 1994; Cuddeford 2001). Drogoul et al. (2001) showed reduction in NDF digestibility from 46.1% to 39.3% as barley (*Hordeum vulgare*) contents increased from 0 to 50% in feed, barley and hay combination, fed to ponies and hypothesized that the reduction is connected with fermentable starch reaching hindgut and resulting in the reduction of number of cellulolytic microorganisms. The exact concentration of starch in the horse diet was

not defined by the Drogoul et al. (2001). Potter et al. (1992) defined the maximal amounts of starch in horse diet up to 3.5 to 4 g/kg BW while Cuddeford (1999) suggested lower amounts; 2 g/kg BW or lower.

The obtained results show an increase in the number of cellulolytic bacteria (Table 4, Table 5, Table 6) and the increase of IVNDFD (Table 8) when increasing the starch content up to 0.9 g/kg BW in horse diet. Moore and Dehority (1993) with an average starch intake of 0.53 kg/day in high concentrate diet showed that higher starch intake did not have a negative effect on *in vivo* cellulose digestibility in ponies. Fiber digestibility of high concentrate (60% hay and 40% concentrate) diet was higher (54.23%) than of high hay (90% hay and 10% concentrate) diet (51.14%). Brown (2006) also had higher ($P<0.05$) fiber digestibility in high concentrate diet (31.2%) compared to high forage diet (27.1%) however, the author discussed that the low fiber digestibility in high forage diet is probably mainly due to the high levels of lignin in high forage diet that decreases the extent of digestion. In this study the observed higher digestibility with higher starch content in horse diet was not due to the lignification of the tested forage samples as the only difference during digestibility determination was the feces used; the same forage samples were tested in all three incubations.

Table 8. Fecal *in vitro* neutral detergent fiber digestibility (IVNDFD) and *in vitro* dry matter digestibility (IVDMD) of three evaluated forages (straw, meadow hay and clover hay) – influence of starch content in three treatments (I: starch 0.3 g/kg BW; II: starch 0.6 g/kg BW; III: starch 0.9 g/kg BW)

	Straw		Meadow hay		Clover hay	
	IVNDFD (%)	IVDMD (%)	IVNDFD (%)	IVDMD (%)	IVNDFD (%)	IVDMD (%)
Treatment I	27.56	22	30.69	36.94	35.44	39.96
Treatment II	38.02	27.79	33.96	37.5	41.96	42.14
Treatment III	39.05	30.09	40.15	41.9	46.11	43.38
P value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Conclusions

Starch increase in the diet up to 0.9 g/kg BW induces proliferation of feces bacteria, primarily cellulolytic bacteria and improves *in vitro* neutral detergent fiber and dry matter digestibility in horses.

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