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Araştırma Makalesi ***Research Article***

Effect of Different Level of Psyllium Supplementation to Horse Diet on *in vitro* Fermentation Parameters and Methane Emission

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Abstract

The purpose of this study was to determine the effect of psyllium addition to horse diets on methane emissions and digestion parameters by *in vitro* digestion technique using horse feces as inoculum. The effect of 0 (control group), 5, 10, 20 and 40 g/kg DM (Dry matter) (treatment groups) psyllium (Psyllium Husk, Solgar, UK) supplementations to horse diet were determined on *in vitro* total gas and methane production, metabolisable energy (ME), organic matter digestion (OMD), ammonia nitrogen (NH_3-N), short chain fatty acids (SCFA) and pH value. *In vitro* digestibility technique was performed with using glass syringes of 100 ml volumes (Model Fortuna, Germany) at 39.0±0.2°C for 24 hour incubation. In the study, *in vitro* total gas production was linearly decreased in treatment groups (up to 130 ml/g DM) compared to control group (181 ml/g DM) ($P<0.001$). Inclusion of psyllium to horse ration decreased methane production up to 35% ($P\leq0.01$). The ME, OMD and SCFA levels of horse diet affected by the psyllium supplementation ($P<0.01$). Addition of psyllium at the levels of 5, 10, 20 and 40 g/kg DM did not have an effect on NH_3-N and pH ($P>0.05$). Consequently, it was demonstrated that psyllium, which use commonly in constipated horses because of laxative efficacy, reduced methane emission as another positive effect in horses. Although psyllium reduced methane emission, it had adverse effects on *in vitro* digestibility of horse ration. However, it was considered that further investigations are necessary to understand the effects deeply by doing the *in vitro* or in vivo digestion trials with lower doses or psyllium is not suitable to use in healthy horses for a long time.

Özet

At Rasyonuna Farklı Düzeylerde İlave Edilen Psilyum'un *in vitro* Fermentasyon Parametreleri ve Metan Salınımına Etkisi

Bu çalışmanın amacı, at rasyonlarına psilyum ilavesinin metan salınımı ve sindirim parametrelerine etkisinin at dışkısı inokulumu kullanarak *in vitro* sindirim tekniği ile saptanmasıdır. At rasyonlarına 0 (kontrol grubu), 5, 10, 20 ve 40 g/kg kuru madde (KM) (deneme grupları) düzeyinde psilyum (Psyllium Husk, Solgar) ilavesinin *in vitro* toplam gaz ve metan üretimi ile metabolik enerji (ME) organik madde sindirimi (OMS), amonyak azotu (NH_3-N), kısa zincirli yağ asitleri (SCFA) ve pH değerlerine etkisi belirlenmiştir. *İn vitro* sindirim denemesi 100 ml'lik cam şırıngalar (Model Fortuna, Almanya) içinde 24 saat süresince 39.0±0.2°C'lik inkübasyonda gerçekleştirilmiştir. Çalışmada, i*n vitro* toplam gaz üretimi kontrol grubuna (181 ml/g KM) göre deneme gruplarında (130 ml/g KM'e kadar) linear olarak azalmıştır ($P<0,001$). Metan üretiminin psilyum ilavesinin artışıyla yaklaşık %35'e kadar azaldığı saptanmıştır ($P\leq0,01$). At rasyonunun ME, OMS ve SCFA düzeyi psilyum ilavesi ile olumsuz etkilenmiştir ($P<0,01$). Sindirim sıvılarının NH_3-N ve pH düzeyi at rasyonlarına 5, 10, 20 ve 40 g/kg KM psilyum ilavesi ile değişmemiştir ($P>0,05$). Sonuç olarak, konstipe atlarda laksatif etkinliğinden dolayı yaygın olarak kullanılan psilyumun, bir başka olumlu etkisi olarak atlarda metan salınımını azalttığı ortaya konmuştur. Ancak, metan üretimini azaltmasına karşın; at rasyonunun *in vitro* sindirilebilirliğini olumsuz etkilediğinden dolayı daha düşük dozlarının *in vitro* veya *in vivo* sindirim denemeleriyle araştırılması ya da sağlıklı atlarda psilyumun uzun süre kullanılmaması gerektiği düşünülmektedir.

Introduction

Laxative feedstuffs (wheat bran, flax seed meal, vegetable oils) or feed supplements (mineral oils) have been used commonly in the diet of constipated horses for preventive purposes (Frape, 2004). Psyllium (seed and husk of *Plantago species,* especially *P. ovata*), which is an effective laxative feed supplement because of branched-arabinoxylan in content, is used as supportive application for prevention of constipation in horse (Hotwagner and Iben, 2007; Washington et al., 1998). In countries that advanced in terms of horse breeding, especially in the United States, have produced commercial feed additives (pasta form; Equine Challenge™ Sand FlushPLUS, USA or granules form; UltraCruz® Equine Sand Clear, USA) produced from psyllium for digestive problems of horses. It has been reported that *Plantago species* (*Plantago afra* and *Plantago arenaria*) was disposal sand in the digestive tract and reduced pain of the colic in horses (Hotwagner and Iben, 2007). Besides that, psyllium is preferred as soluble dietary fiber source for cat-dog and human diets in the United States due to hypoglycemic, hypocholesterolemic and laxative activity (Fischer et al., 2004).

Plantain species, which are plants belonging to the *Plantaginaceae* family, are commonly found in grasslands had warm climates, can grow up to 10-30 cm. These species are located and grow in many countries around the world (Samuelsen, 2000; Stewart, 1996). This plant family can grow easily in all kinds of soil types (pH 4.2-7.8), but mostly in sandy and moistly soil (Stewart, 1996). In Turkey, the narrow-leave (*P. lanceolata*) and broad-leave (*Plantago major*, and *Plantago asiatica*) species of plantain are widely found (Kara et al., 2015b; Samuelsen, 2000). The leaves of this plant species have been used as traditional from ancient civilizations (Egypt and Greek) due to treatment influences on various disorders of biological active compounds in their content. (Samuelsen, 2000). Plantago leaves are consumed greedily by grazing animal species because of the palatability (sorbitol accumulation), especially in salty and arid soil conditions (Fons et al., 1998; Stewart, 1996). Plant seeds are quite small (average 0.6 × 1.2 mm) and slightly bitter. These plants grow extensively in sandy soil lands and leaves are horizontal as parallel with the soil. Therefore, horses which are grazing in plantago grasslands may facilitate the consumption of sand together with the plant. However, the sand accumulation in digestive tract can be naturally removed with feces through the plant's laxative effect.

Total anthropogenic green house gas emissions (the development of agriculture-livestock and industrial) increased significantly at last 40 years according to the International Climate Change Panel (IPCC, 2014). Total anthropogenic GHG emissions were the highest in human history from 2000 to 2010 (IPCC, 2014). The 50-60% of global methane emissions comes from agriculture and livestock (especially dairy cattle, beef, sheep) production (Ellis et al., 2007). Therefore, it is important to determine the methane concentration produced in the digestive tract of horse.

In recent years, it was carried out numerous digestion trials in ruminants, *in vitro* (Kara, 2015; Kara et al., 2015a; Kara et al., 2015b; Kara et al., 2015c). Number of *in vitro* digestion trials in horses has increased in the last few years (Almeida et al., 2012; Kholif et al., 2015; Santos et al., 2012; Sweney, 2012). Intestinal content from different parts of the intestine (cecum, colon) or feces were used in digestibility trials conducted in horses *in vitro*. But, feces inoculum provides advantage due to easy availability compared to the intestinal content and it does not require intestinal cannulation of horse. In a previous study, the *in vitro* gas production performed using the feces inoculum was reported similar to that of made with cecum inoculum (Santos et al., 2012).

There are many *in vitro* digestion studies conducted in ruminant about reduction emissions of animal-related greenhouse gas (especially methane). Horses produce also methane at certain levels in the digestive tract although not as much ruminants. The aim of the present study was to determine the effect of psyllium on *in vitro* methane emission and digestive parameters using feces as inoculum.

Materials and Methods

The Psyllium Husk

In this study, psyllium supplement was used as a commercial product (Psyllium Husk Fibre, Solgar, UK).

The Chemical Analysis of Feed

The total mix diet of horse was milled through a 1 mm sieve (IKA MF10.1, Germany) for use in chemical analysis and *in vitro* gas production. Dry matter (DM) (method 14.081), crude ash (CA) method 942.05), crude protein (CP) (method 954.01), diethyl ether extract (EE) (method 920.39), and crude fiber (CF) (methods 7.066-7.070) compositions of the diet were analyzed using the AOAC methods (AOAC, 1995). The neutral detergent fiber (NDF) contents were analyzed by using a fiber analyzer (Velp FIWE3, Italy) according to the methods reported by Van Soest et al. (1991). The NDF was determined using sodium sulfide (Merck) and thermo-stable α-amylase (Megazyme, Ireland)(aNDF). The NDF did not contain ash residue (aNDFom). Non-fibrous carbohydrate (NFC) levels were calculated using the

following formula (NRC, 2001): NFC % = 100 – (aNDFom% + CP% + EE% + CA%). The ingredient and nutrient matter composition of the diet are presented in Table 1.

Table 1. Ingredients and analysis values of adult horse (quarter horse) diet used in the present study.

Feedstuff	**%**
Meadow hay	41.00
Wheat straw	27.00
Oat	16.00
Wheat bran	8.00
Flax seed meal	7.00
Di calcium phosphate	0.30
Salt	0.70
Analysis values (in DM)	
DM (as feed basis)	90.13
CP	10.62
EE	3.35
CA	10.70
CF	15.21
aNDFom	34.29
NFC	41.04
TCar (mg/kg)	13.51

DM: dry matter, CP: crude protein, EE: diethyl ether extract, CA: crude ash, CF: crude fiber, NDF: ash-free neutral detergent fiber, NFC: non fibrous carbohydrate, TCar: total carotenoid.

The total carotenoid composition of diet was determined as basis to method of Biehler et al. (2010). For this, about 2 g of samples weighed into a 15-mL conic centrifuge tube (Isolab, Wertheim, Germany). 5 ml of methanol and 1 ml of 30% methanolic potassium hydroxide was then added into tube and was mixed using a vortex at 2000 rpm for 30 minutes (Velp Vortex ZXClassic, Velp, Italy), and incubated in thermostatically controlled cabinet +4°C (Lovibond, Dortmund, Germany). After incubation, tubes were centrifuged a cooling centrifuge (Nüve NF800R, Nüve, Turkey) at 4000 rpm and at +4ºC for 5 minutes. Supernatant was decanted into a 50-mL conic centrifuge tube (LP Italiana SPA, Milano, Italy). Extraction procedure was repeated twice with 8 mL of hexane, and extracts were pooled. On top of pure and separate extracts, 25 ml of saturated aqueous sodium chloride was added and the mixture shaken. Pooled extracts were read exactly for volume determination. The absorbance values of extracts were measured using UviLine 9100 spectrophotometer (SI Analytics, Germany) at 450 nm. Carotenoid contents were estimated by using the following formula:

$$\text{Concentration (mg/kg)} = (A^* V^* 10^4) / (A^{1\%}{}_{1cm}{}^* W)$$

A= absorbance, V= volume of extracts (ml), $A^{1\%}{}_{1cm}$= Absorption coefficient, W= Sample weight

***In vitro* Digestion Technique**

Feces samples used as an inoculum in the current study obtained from two thoroughbred horses (6-7 years of age, 480-500 kg of body weight) fed a ration containing 70% roughage and 30% concentrate feed, in DM basis. Feces samples were collected soon after defecation and transferred into a thermos containing water at 39°C under CO_2 gas and transferred to the laboratory within 1h. Feces samples were diluted at 1:10 ratio with 0.9% sterile serum physiologic solution (Polifleks, Polifarma, Turkey) using a laboratory type blender (Waring Products Division, Torrington C.T., USA). Diluted feces inoculum was filtered through four layers of cheese cloth under constant CO_2 gas (anaerobically) and used in the *in vitro* digestion technique.

The *in vitro* digestion technique was carried out in glass syringes with 100 ml volume (Model Fortuna, Haberle Labortechnik, Germany) using the mediums prepared according to Sunvold et al (1995) and Sweney (2012). Medium mixture used in the present study is given in Table 2.

The diet samples (500±10 mg as DM) were incubated with medium mixture (30 ml) and feces inoculum (5 ml) in glass syringes as triplicate. The syringes were closed using clips and then the initial volume recorded and incubated in a water bath with thermostat (Yapar Stainless Steel, Turkey) at 39.0±0.2°C up to 24h. In addition, three blank syringes (no template; medium mixture + feces inoculum) were used to calculate the total gas production.

Determination of Total Gas and Methane Production

In this study, the total gas volume (ml) was determined from the calibrated scale on the syringe at the end of 24 h incubation. After measuring the total gas volume at 24 h, the plastic syringe outlet was inserted into the inlet of the methane analyzer (Sensor, Europe GmbH, Erkrath, Germany) using three way stop cock (Polycarbonate, Mediplus, India) and the piston was pushed to insert the accumulated gas into the analyzer. A micro filter with 0.22 µm pore diameter (MillexGP Filter Unit, Merck Millipore Express Membrane) was used during the injection of the total gas. The methane as a percent (%) of the total gas was displayed on a PC (Vestel, Turkey). The methane level was converted to ml by the following formula (Kara 2015).

In vitro methane production (ml) = [(in vitro total gas production, ml)*(*in vitro* methane production, %)]/100

Table 2. Composition of *in vitro* fermentation medium.

Component	Amount
mL/L	
Solution A[a]	330.0
Solution B[b]	330.0
Trace mineral solution [c]	10.0
Water-soluble vitamins [d]	20.0
Folate: biotin solution [e]	5.0
Riboflavin solution [f]	5.0
Hemin solution [g]	2.5
Short chain fatty acids [h]	0.4
Resazurine [i]	1.0
Distilled H_2O	296.0
g/L	
Yeast extract	0.5
Trypticase	0.5
Na_2CO_3	4.0
Cystein HCl*H_2O	0,5

[a]Composition (g/L): NaCl, 5.4; KH_2PO4, 2.7; $CaCl_2*H_2O$, 0.16; $MgCl_2*6H_2O$, 0.12; $MnCl_2*4H_2O$, 0.06; $CoCl_2*6H_2O$, 0.06; $(NH_4)_2SO_4$, 5.4.

[b]Composition: K_2HPO_4, 2.7 g/L.

[c]Composition (mg/L): ethylene diamine tetraacetic acid (disodium salt), 500; $FeSO_4*7H_2O$, 200; $ZnSO_4*7H_2O$, 10; $MnCl_2*4H_2O$, 3;H_3PO_4, 30; $CoCl_2*6H_2O$, 20; $CuCl_2*2H_2O$, 1; $NiCl_2*6H_2O$, 2; $Na_2MoO_4*2H_2O$, 3.

[d]Composition (mg/L): thiamin-HCl, 100; d-pantothenic acid, 100; niacin, 100; pyridoxine, 100; *p*- aminobenzoic acid, 5; vitamin B_{12}, 0.25.

[e]Composition (mg/L): folic acid, 10; d-biotin, 2; NH_4HCO_3, 100.

[f]Composition: riboflavin, 10 mg/L in 5 mmol/L of Hepes.

[g]Hemin: Hemin 500 mg/L of 10 mmol/L NaOH

[h]Composition: *n*-valerate, isovalerate, isobutyrate and DL alpha- methylbutyrate, 250 mL/L

[i]Composition: 1 g resazurine/L distilled water

Estimation of Metabolisable Energy (ME), Organic Matter Digestibility (OMD) and Short-Chain Fatty Acids (SCFA) Levels

In the study, ME and OMD values of horse diet samples were calculated according to following equations as reported by Kholif et al. (2015) and developed by Menke et al. (1979).

ME (MJ/kg DM) = 2.20+0.136×GP+0.057×CP

OMD (DM%) = 14.88+0.889×GP+0.45×CP+0.0651×CA

Short-chain fatty acids (SCFA) were calculated according to method described by Getachew et al. (2002) as:

SCFA (mmol/200 mg DM) = 0.0222 GP–0.00425

The GP is net gas production at 24 h (ml/200 mg DM), and CP and CA are crude protein and crude ash (DM%), respectively

Determination of Ammonia-N (NH_3-N) and pH in Digestion Fluid

The pH of digestion fluid which was collected at the end of 24 h incubation was determined using a digital pH meter (Seven Compact pH/Ion S220, Mettler Toledo, Switzerland). The ammonia-N (NH_3-N, mg/dL) concentration of the same digestion fluid was determined with distillation (Velp Distilation Unit, Italy), without acid digestion and after distillation with potassium hydroxide in boric acid and titration using diluted hydrochloric acid (0.2 N).

Statistical Analysis

Statistical analysis of the study data were performed using SPSS 17.0 software (IBM Corp., Armonk, NY, USA). One-way variance analyses (ANOVA) were implemented for homogeneous variances by General Linear Model procedures to test treatment differences. Data was analyzed based on the statistical model: Yij= µij +Si+ei. Where, Yij= the general mean common for each parameter under investigation. Si= the effect of psyllium on the observed parameters, ei= the standard error term. The linear and quadratic polynomial effect levels of psyllium at different levels on researched parameters were analyzed by using described polynomial contrast tests. P-values<0.05 were considered statistically significant different.

Results

The results of crude nutrient matter composition analyses of horse diet was given in the Table 1. *In vitro* total gas production of control group was determined as 181 ml/g DM at 24-hour incubation; the values in treatment group were ranged from 130 to 175 ml/g DM. *In vitro* total gas production were decreased as linear (P<0.001) and quadratic (P<0.01) after the 5, 10, 20 and 40 g/kg DM supplementations of psyllium to horse diet. *In vitro* methane production was decreased linearly with the increase of psyllium supplementation (P=0.01). Estimated ME, OMD and SCFA levels of horse diet decreased as linear (P<0.001) and quadratic (P<0.01) at significant ratio with psyllium supplementation. In the current experiment, NH_3-N and pH levels of the digestion fluids were not influenced by psyllium supplementation at 5 to 40 g/kg DM ratio to horse diet (P>0.05) (Table 3).

Discussion

Plantago species were used as forage for ruminating animals and horses (Deaker et al., 1994), as well as extruded diet of pet animals, because of the carbohydrate content in the husk and seed of these plants have added as a dietary fiber source (Swanson et al., 2001). Besides, due to laxative efficacy of plantago

husk (psyllium; especially *Plantago ovata*) can be used in diets of horses which have constipation risk or constipated (Hammock et al., 1998; Hotwagn and Iben, 2007).

Table 3. Effect of different level of psyllium supplementation to horse diet on *in vitro* fermentation parameters.

Parameters	Control Group	Psyllium Supplementation (Treatment Groups)				SEM	P-Values	
		5 g/kg	10 g/kg	20 g/kg	40 g/kg		Linear	Quadratic
TGP	181.03	175.07	177.70	159.03	130.67	5.30	<0.001	0.004
Methane	0.66	0.58	0.59	0.47	0.43	0.03	0.010	0.843
ME	7.75	7.58	7.66	7.15	6.38	0.14	<0.001	0.004
OMD	58.98	57.92	58.39	55.07	50.02	0.59	<0.001	0.004
NH_3-N	61.00	65.98	65.66	64.92	67.13	1.14	0.271	0.654
SCFA	0.79	0.77	0.78	0.70	0.57	0.02	<0.001	0.004
pH	5.91	6.30	6.43	5.87	6.97	0.15	0.076	0.458

ME: Metabolisable energy, MJ/kg DM
Methane: Volume of *in vitro* methane produced at 24 hours incubation, mL/g DM
NH_3-N: Ammonia nitrogen, mg/dL
OMD: Organic matter digestibility, %
SCFA: Short chain fatty acid, mmol/0.2 g DM
TGP: Volume of *in vitro* gas produced at 24 hours incubation, mL/g DM
SEM: Standard error means

Many studies about the reduction of methane emissions originated from animal production (especially ruminant rearing systems) have carried out at the present time. Share in global methane emissions of cattle has demonstrated in previous studies (Kamalak et al., 2005; Kara, 2015; Kara et al., 2015a; 2015b; 2015c). Despite the fact that the dairy cattle produce annually 35-55 kg methane, the adult horse produces 18 kg methane, annually. Considering the horse population on the world, the share of horses in animal-derived methane is at a rate of 0.5-3% (Crutzen et al., 1986). The rate in globally methane emission of horse-derived methane emission have significantly increased in the last 10 years because of the increase in horse population in the world (Rafiu et al., 2012). The previous studies did not demonstrate that methane emissions in horses can be reduced by diet manipulations. In the present study, psyllium supplementation at 5, 10, 20 and 40 g/kg DM to horse diet reduced *in vitro* methane emission until 35%. In the previous study, researcher stated that ruminal methane production reduced by *Plantago lanceolata,* especially leaf parts, as *in vitro* (Kara et al., 2015b). The same researchers has expressed that "the leaf of *Plantago lanceolata* can be used as an anti-methanogenic feed/feed additive due to its effect of reducing to ruminal methane emissions". In the current study, because of the reducing activity as *in vitro* horse-methane emission of psyllium can be regarded as anti-methanogenic diet additive for horse.

In vitro gas production level is changed by the nutrient composition of tested feedstuff (cell wall substances, starch, sugar, etc.), the presence of compounds inhibiting gas production (such as condensed tannins, polyethylene glycol), the diet of donor animal's diet, the microflora and microfauna of fermentation inoculum (rumen fluid, intestinal content, feces) and the quality of fermentation provided (Goel et al., 2008; Hook et al., 2010). In the present study, the *in vitro* total gas production of horse diet ration was identified as about 181 ml/g DM at 24-hour incubation. Kholif et al. (2015) determined that the horse diet produced 225 ml/g DM total gas at 24 hour incubation in the *in vitro* digestion experiment which was carried out using the horse feces and according to the gas production technique of Theodorou et al. (1994). Almeida et al. (2012) demonstrated that the hay produced about 75 ml/g total gas at 24 hour incubation with a semi-automatic gas production system using the inoculum prepared of dorsal colon content in horses.

In the present study, *in vitro* total gas production decreased linearly up to 28% depending on psyllium supplemented to diet. Previous *in vitro* digestion study carried out using dog feces inoculum demonstrated that psyllium seed had low gas production and OMD values and slowly fermented (Calabro et al., 2013). It was found that ME, OMD and SCFA values decreased *in vitro* total gas production. In the present study, reducing of *in vitro* feed digestion may be associated with high soluble

dietary fiber content and water absorption activity of psyllium (Cannon, 2009). Increasing viscosity of *in vitro* digestion environment (gas production syringe) may be diminished or stopped effectivity of microbial enzymes on substrate (horse diet samples) and then gas production values may be reduced.

Consequently, it is considered that constipation and methane production in horses can be decreased by feeding psyllium supplemented diet. However, it can be concluded that psyllium is not useful in healthy horses for the long-term due to negative effect on the *in vitro* gas production, ME, OMD, and SCFA values.

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