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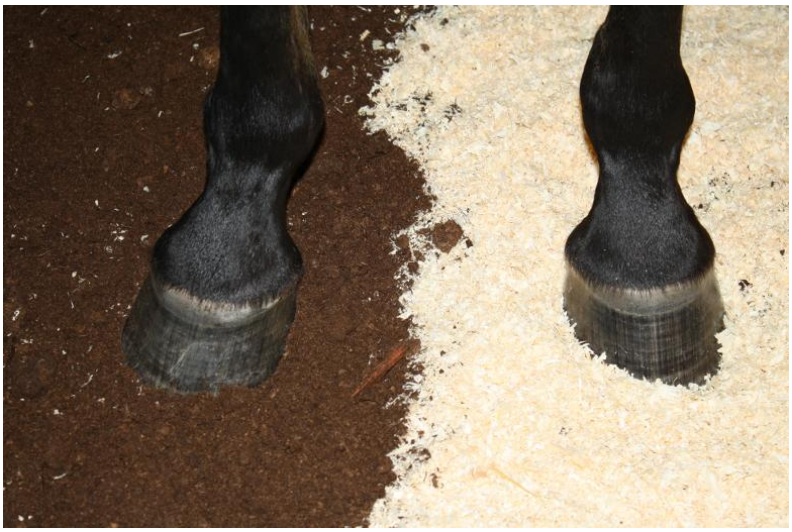

Institutionen för husdjurens utfodring och vård

Examensarbete 295
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Swedish University of Agricultural Science
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Louise Hübinette

Handledare: Anna Jansson
Examinator: Jan Erik Lindberg

Nyckelord: horses, faecal microflora, faecal pH, bedding material, wood shavings, sphagnum peat

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Contents

Abstract

The main purpose of this study was to determine the impact of bedding material on the faecal microflora of horses. Another objective was to investigate the hygienic and physical qualities of the bedding materials used and how these may change in the course of the trial period. The bedding materials used were sphagnum peat and wood shavings. Six horses had each material as bedding for a three week period in a change–over experiment. The horses' ration of roughage was distributed directly on top of the bedding. Faecal samples were collected weekly for measuring of pH and for Terminal restriction length polymorphism (T-RFLP) analysis. T-RFLP analysis is a PCR based method were the size of 16S rRNA fragments are determined in order to study microbial communities. Samples of bedding material were collected at the start up an end of each period and sent for hygienic analysing. Samples of the haylage fed to the horses were collected weekly and sent for analysis as one sample per period. Samples were also taken from both materials in the beginning and end of each period for determination of pH, dry matter and water holding capacity. Registrations of indoor and outdoor temperature and humidity were done on a daily basis.

Bedding material did not influence the faecal pH. The T-FRLP analysis did not show any impact from bedding material on the microflora. Peat had significantly higher water holding capacity compared to wood shavings. *Penicillium* spp. was the most common mould found in peat and wood shavings. There was no difference in number of aerobic bacteria, yeasts, moulds and thermophilic fungi between peat and wood shavings. The number of aerobic bacteria had increased significantly in both bedding materials in the end of the periods. In conclusion, bedding material does not influence the faecal microflora with the conditions given in this study.

Sammanfattning

Studiens huvudsyfte var att undersöka strömaterialets inverkan på hastens fekala mikroflora. Även strömaterialens hygieniska och fysikaliska egenskaper och hur dessa förändrades under försökets gång undersöktes. Strömaterialen som användes var vitmossetorv och kutterspån. Sex hästar fick vardera strömaterial i sin box i tre veckor i ett change-over-försök. Hösilage utfodrades direkt ovanpå ströbädden. Träckprover togs efter varje vecka för mätning av pH och analys med T-RFLP. T-RFLP är en PCR baserad metod där 16S rRNA fragment storleksbestäms för studie av mikrobiella samhällen. Prover av strömaterialen togs i början och i slutet av varje period och skickades för hygienisk analys. Prover togs varje vecka av det hösilage hästarna utfodrades med och skickades för hygienisk analys som ett samlingsprov per period. Prover togs även av strömaterialen i början och slutet på varje period för bestämning av pH, torrsubstans och absorptionsförmåga. Mätningar gjordes dagligen av temperatur inne och ute samt luftfuktighet inne.

Strömaterialet påverkade inte fekalt pH. T-FRLP analysen visade ingen påverkan av strömaterialet på mikrofloran. Torvströ hade signifikant bättre absorptionsförmåga än kutterspån. *Penicillium* var det vanligaste förekommande mögelsläktet i både torv och spån. Det var ingen signifikant skillnad mellan strömaterialen vad gäller antal aeroba bakterier, jästsvampar, mögelsvampar och termofila svampar. Antalet aeroba bakterier i strömaterialen ökade signifikant från början till slutet av försöksperioderna. Sammanfattningsvis påverkar inte strömaterialet den fekala mikrofloran under de förhållanden som givits i denna studie.

Introduction

Wood shavings and peat are two bedding materials commonly used in Sweden (Airaksinen *et al*., 2005). Horses are often fed with roughage directly on top of the bedding material, making it plausible that they may consume small amounts of their bedding. Horses that are not fed *ad libitum* may also consume some of their bedding due to high motivation for feeding. The chemical composition of peat and wood shavings differ and if the horse consumes some of its bedding changes in the microbial ecosystem of the gastrointestinal tract might occur.

Studies have shown the properties and hygienic qualities of different bedding materials. It is however unclear how different materials will be influenced and changed microbiologically in a horse stable environment.

The main objective of this trial was to determine if the bedding material has an impact on horse's faecal microflora. Another objective was to investigate the hygienic quality of the bedding materials and quality changes that may occur in the environment of a horse stable.

Literature review

Sphagnum peat

Sphagnum peat is a type of soil consisting of decaying *Sphagnum* plants. In addition to water, sphagnum peat is composed of cellulose, lignin (20-30 %), humic acid (5-20 %) and colloidal humic substances (Crum, 1988). Peat moss has a pH of 3.7 to 4.2. Peat has a high capacity of holding water and ammonia absorption (Airaksinen *et al*., 2001). The ammonia undergoes nitrification to form nitrates, a process performed by bacteria present in soil (Crum, 1988). The levels of ammonia and dust have been found to be low when peat has a humidity of about 50 %, but there was a 10-fold increase of dustiness when humidity was decreased from 50 to 30 % (Larsson *et al.*, 1999).

Studies have shown that *Penicillium* spp. is the most common fungi found in peat (Airaksinen *et al*., 2001; Larsson *et al.*, 1999). Lappalainen (2002) showed that airborne fungal concentrations were somewhat higher when peat was used as bedding compared to wood shavings. A heterogeneous quality, dark colour and dustiness have been described as disadvantages of peat as bedding material (Airaksinen *et al*., 2001).

Wood shavings

Wood shavings together with sawdust are the main by-products of the lumber industry. Wood shavings are created when lumber mills planes dried rough wood into smooth wood. It has a high dry matter content making it suitable as animal bedding material when dust extracted. Dry wood is composed of cellulose (~50 %), lignin (~16 to 33 %), hemicelluloses and other substances such as tannins, oils and fats and 0.2 to 1.0 % inorganic compounds (Miller, 1999).

Wood shavings have been suggested as an alternative bedding material to straw for horses that have allergies or chronic disorders of their airways (Fleming *et al.*, 2008). Fleming *et al*. (2008) confirmed in their study that the particle concentration in the stable air was reduced

when wood shavings were used as bedding instead of straw. The hygienic quality of wood shavings were higher than straw but poorer compared to paper cuttings.

Wood shavings do not posses as good water holding capacity and ammonia absorption as peat. Wood shavings do however contain lower number of fungi and bacteria compared to peat (Airaksinen *et al*., 2006).

Horse stable environment

Horse stables often rely on non mechanical ventilation and during winter conditions it is not uncommon for the doors of the stables to be left closed, minimizing the ventilation (Elfman et al., 2009; Wheeler et al., 2001). This contributes to high humidity which is a favourable condition for mould growth. The moderate temperatures and availability of nutrients are also favourable conditions for bacterial growth (Bey et al., 2002). Choice of bedding material and mucking regimen has a large influence on air quality and hygiene of the stable (Fleming et al., 2009).

Bedding material and fodder are the major sources for microorganisms found in stables (Elfman et al., 2009; Airaksinen et al., 2001). Considering that high humidity is not uncommon, mould growth on the interior is also plausible (Elfman et al., 2009). There are several studies on airborne particles present in horse stables. The most common microorganism pollutants of the air in the stable are fungi, bacteria, yeasts, viruses and mites (Fleming et al., 2008). The microorganisms present differ with the bedding material used but among the most common fungi are *Aspergillus fumigatus*, *Penicillium*, *Cladosporium* and *Alternaria* spp. (Elfman et al., 2009; Airaksinen, 2006; Frape, 2004).

Horses´ Gastrointestinal Microflora

Horses are depending upon intestinal bacteria for digestion of cellulose, hemicellulose, pectin, fructo- and galacto oligosaccharides (Frape, 2004). The dietary composition has a large impact on the microbial ecosystem inhabiting their gastrointestinal (GI) tract. Changes in horses' diets will lead to changes of the microbial population. Bacteria constitute more than half the dry weight of equine faeces and the number of bacteria in the digestive tract outweighs the total number of tissue cells in the body tenfold (Frape, 2004). The digestion in the caecum and ventral colon is almost entirely carried out by bacteria and ciliate protozoa. This is also where the largest bacteria populations of the large intestine are found. Large variations in the number of specific microorganisms can occur due to changes of available nutrients and pH.

Muhonen (2008) studied the impact of crude protein content and conservation method on abrupt feed changes and metabolism and hindgut ecosystem. Horses were fed a high crude protein diet (>40 % of the recommended intake) and a diet with the recommended intake of crude protein. During the first 24 hours after switching diet, the total concentration of lactobacilli and anaerobe bacteria in the colon were higher on the high protein diet than the regular protein diet. Between day 7 and 22, the concentration of volatile fatty acid (VFA) was higher and pH lower in the colon on the high protein diet compared to recommended protein diet. An abrupt feed change from hay to silage and haylage were also studied. There were no changes in bacterial counts or VFA in the colon during the first 28 hours following the feed change. There was an increase in water in the colon and changes in number of lactobacilli and streptococci concentrations.

Horses on a high crude protein diet had higher concentration of VFA in colon and lower pH than those fed a diet with recommended amount of crude protein diet.

T-RFLP analysis and effects of diet

Terminal restriction fragment length polymorphism (T-RFLP) is an analysis technique were microbial communities are studied based on differences in the 16S rRNA gene (Zoetendal *et al.*, 2004, Spiegelman *et al.* 2005). The method can be used to study changes in the microbial community due to different factors. DNA is extracted and the 16S rRNA gene is amplified using primers where one is fluorescently labeled. The PCR product is digested with a restriction enzyme. The fluorescently labeled terminal restriction fragments are then separated through an electrophoresis platform creating a digital profile with peaks representing fragment length and abundance. The method is useful for comparing communities and determining species diversity.

Only a few studies have been performed on the faecal microflora of horses with the use of T-RFLP. Willing *et al.* (2009) showed that when performing cluster analysis, samples group together according to different diets and individuals.

Ringmark (2008) performed a feeding trial where 7 horses were fed two different haylage diets in a switch-back study. Studies of T-RFLP patterns showed that the diets impacted the microbiological profile in the colon differently.

Faecal pH

Faecal pH is affected by the horse's diet making it useful for observing differences in feeds. In a feeding trial performed by Medina et al. (2002), associations were found between alterations of microbial profile and changes in colonic and cecal pH. In the study, horses were fed either a high-starch diet or a high-fiber diet. Horses fed the high-starch diet had a mean cecal pH of 6.85 and a colonic pH of 6.79. The horses fed the high-fiber diet had a mean cecal pH of 7.15 and a colonic pH of 7.14.

In a feeding trial performed by Julliand et al. (2001), an increase of barley in the diet lowered the pH in the cecum and right ventral colon. Berg *et al*. (2005) found that faecal pH decreased when horses were fed diets supplemented with fructo-oligosaccharides, due to an increase in production of lactic acid and short chain fatty acids.

Horses fed diets with high protein content (160 % of recommended intake) have lower faecal pH as well as higher content of water in the faeces, compared to horses fed a diet containing the recommended amount of protein (Connysson *et al*., 2006).

Faecal pH will also be affected with the transition from one diet to another. Van den Berg et al. (2006) showed that faecal pH increased significantly on day 2-4 when switching from pasture to conserved forage with an increasing ratio of concentrate. The pH also increased when switching back to the forage diet again.

The feeding sequence may impact faecal pH as reported in a study where the horses had higher pH when fed hay 30 minutes prior to oats, compared with the reversed feeding strategy (Zeyner et al., 2004). The feeding frequency, amount of exercise and type of bedding does not impact faecal pH (Williamson et al., 2007).

Experimental Work

Several studies have shown that the diet has a large impact on horses' gastrointestinal microflora. It is however not known whether the bedding material has an impact. The aim of this study is to find whether the bedding material influences the microflora of the horse or not. The purpose was also to determine the physical properties of the materials and how these may change in the environment of a horse stable.

Material and Methods

Animals, management and experimental design

Six privately owned horses were included in the experiment, four ponies and two Norwegian Fjord Horses. Before the experimental started, a mixture of wood shavings and sawdust were used as bedding material. The horses were kept inside from about 4 p.m. to 8 a.m. in individual box stalls at about 3x3 m^2 each. The rest of the day they were kept outside in paddocks. They were all fed the same haylage but different types of concentrates. The haylage was given directly on top of the bedding. The rations differed from horse to horse but were consistent for every horse throughout the experimental period.

The experiment was performed as a three week change-over experiment and was performed during 6 weeks (42 days) from April to May of 2009. Collections of faecal samples were carried out in the mornings of day 0, 8, 14, 21, 28, 35 and 42. Faecal samples were collected immediately from the floor in 50 ml Falcon tubes which were filled up to minimize the presence of air. Each sample was collected from several places of the pile of droppings to gain a representative selection. Samples for pH measuring were collected in plastic bags as larger amounts were needed. Faecal samples were stored in – 20 °C.

Day 0 to day 21 is referred to as period 1 and day 21 to 42 as period 2. Horse 1, 2 and 3 had sphagnum peat as bedding material for the first period while horse 4, 5, and 6 had wood shavings. In the second period the bedding materials were reversed. The wood shavings used were dust free and bagged in plastic, each bale weighing approximately 25 kg. The sphagnum peat used was bagged in bales of about 35 kg, which equals 300 l.

The indoor and outdoor temperatures as well as humidity were registered daily in the mornings. The indoor temperature and the humidity were measured next to one of the stable doors and in a corner of one of the box stalls, right above the bedding. The outdoor temperature was measured outside one of the stable doors.

The water holding capacity, pH and dry matter were measured for both bedding materials in the experimental start ups and after each period. Samples of bedding materials were collected in the beginning and end of each testing period and sent to the National Veterinary Institute (SVA) for microbiological analysis (Nordic Committee on Food Analysis, No 98, 4th Ed., 2005). The analysis conducted were determination of cfu (colony-forming units) level and water activity and mould flora identification.

The samples collected in the beginning of the periods were taken directly from newly opened bales of bedding material. The samples collected at the end of the testing periods were taken from a specific corner of each box stall where the bedding was subjected to minimum

handling and where no new bedding material was added. The amount for one sample was about 1 l.

Samples of haylage, about 2 - 3 l per sample, were collected once a week and stored in – 20 °C. After each period a mixture of the three samples from that period were sent to SVA for microbiological analysis. The samples were collected from different parts of the round bale from which the horses were being fed at the time being.

Dry matter and water holding capacity of bedding materials

Samples of the bedding materials were dried for 16 hours in an oven (Memmert, 854 Schwabach, W-Germany) at 110 °C. The water holding capacity was measured in two ways. In the first method (A) a beaker was filled with a layer of bedding material that was two centimetres high when a 5 kilo weight was placed on top. Then 400 ml of water with a temperature of 37 °C was poured into the beaker. The non-absorbed water was poured in to graduated cylinders after five minutes. In the second method (B), 100 ml of 37 °C warm water was poured over 100 ml of bedding material placed in a funnel with filter paper. The non absorbed water was measured in a graduated cylinder.

pH measurements

The pH-value for bedding materials and faecal samples were measured with PHM210 Standard pH Meter (Radiometer Analytical). The pH meter was calibrated with two buffers before measuring. Four grams of peat were mixed with 12 ml of double-distilled water (ddH_20) and 2 grams of wood shavings were mixed with 20 ml of ddH_20. Faecal pH was measured from samples taken after each test week. Fluid was squeezed from fresh faecal samples. Measurements were performed twice for every sample and mean values were calculated.

T-RFLP analysis

DNA was isolated from 300 mg of each faecal sample using the FastDNA® SPIN Kit for Soil and the FastPrep® Instrument (MP Biomedicals, Santa Ana, CA) according to the manufacturer's instructions. Duplicate DNA extractions were performed.

In order to determine that DNA had been successfully isolated, universal 16S primers (Invitrogen) were used to amplify bacterial 16S rRNA genes. The reactions were carried out in 0.2 ml tubes with illustra™ puReTaq Ready-To-go PCR beads with the Bio-Rad MyCycler™ (version 1.065) thermal cycler system. The following cycling conditions were used: 94 °C for 30 sec, 49 °C for 30 sec, 29 cycles x 72 °C for 2 min, 72 °C for 10 min. The PCR-products were marked with a fluorescent compound and the quality was checked on 1 % agarose gel.

Then 16S rRNA genes were amplified from the DNA products with the primers Bact-8F (5'-AGAGTTTGATCCTGGCTCAG-3'), marked with 6-carboxyfluorescein on the 5'end and the reverse primer 926r (CCGTCAATTCCTTTRAGTTT-3' (Invitrogen). The PCR was again carried out in 0.2 ml tubes with illustra™ puReTaq Ready-To-go PCR beads and with the following cycling conditions: 94 °C for 40 sec, 55 °C for 40 sec, 72 °C for 60 sec x 35, 72 °C for 7 min.

Restriction digest was performed using 6 µl of PCR-product, 0.25 µl HAEIII enzyme, 2.5 µl 10x NE Buffer and 16.25 µl H20, at 37 °C for 2 hours.

The samples were diluted 10-fold with ddH_20 and then sent to the Rudbeck Laboratory for analysis with ABI3730 capillary sequencer. Peak Scanner Software version 1.0 (Applied Biosystems) was used to process the results. Results are shown as peaks representing amount of bacteria and number of base pairs (bp). Peaks below a threshold value of 0.5 % were excluded from further analysis. The data was processed in a matrix for cluster analysis using the Bray Curtis similarity tree.

Statistical analysis

The statistical analysis of pH, dry matter, absorption, aerobic bacteria and hygienic quality was performed with Statistical Analysis System (SAS) 9.1 and the General Linear Models Procedure (GLM). A significance level of $p<0.05$ was used for differences between results and differences with $p<0.1$ were considered as tendencies. Values are presented as means +- standard deviation.

Model used for faecal pH: $Y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_k + \delta_l + e_{ijkl}$
Where Y_{ijkl} = observation, μ = mean, α_i = individual, β_j = week, γ_k = period, δ_l = bedding material and e_{ijkl} = random error.

Model used for physical properties of bedding materials: $Y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_k + e_{ijk}$
Where Y_{ijk} = observation, μ = mean, α_i = sample, β_j = period, γ_k = bedding material and e_{ijk} = random error.

Model used for microbiological quality of bedding materials: $Y_{ij} = \mu + \alpha_i + \beta_j + e_{ij}$
Where Y_{ij} = observation, μ = mean, α_i = period, β_j = bedding material and e_{ij} = random error.

Results

Temperature and humidity

The indoor temperature ranged from 3.0 to 11.8 °C and the outdoor temperature from 0.5 to 12.5 °C. The indoor and outdoor temperatures were significantly higher in period 2. The outdoor temperature was also significantly different between days. The humidity ranged from 38 to 71 % outdoors and from 47 to 86 % indoors. There were no significant differences in humidity between periods or days.

Faecal pH

The horses' faecal pH-values ranged from 5.80 to 6.76 (Figure 1). Horse, week and period had a significant impact ($p<0.05$) on the pH-value. Bedding material did not have a significant impact and there were no significant interactions between week and bedding material. The average pH value (mean ± standard deviation) was 6.12 ± 0.22 when peat was used as bedding material and 6.16 ± 0.28 when wood shavings was used.

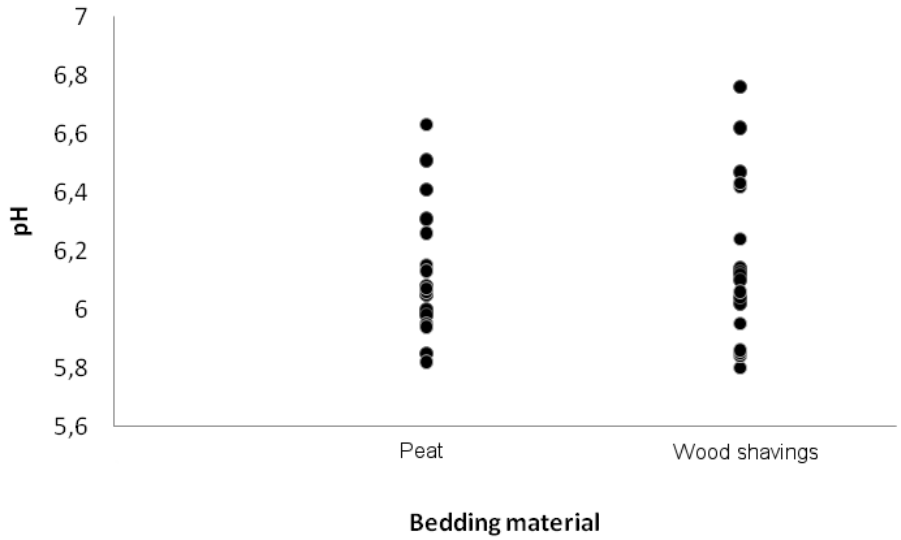

Figure 1. Faecal pH-values measured on fresh faecal samples collected weekly from six horses with peat and wood shavings as bedding material.

T-RFLP

Fragments ranging from 62 bp to 420 bp were detected. The Bray Curtis similarity tree shows that samples from one individual often cluster together (Figure 2). Horse A was an exception of this. In general, there was no clear grouping related to bedding material. An exception of this is horse C where samples from period 1 and 2 clusters together in clearly separated groups.

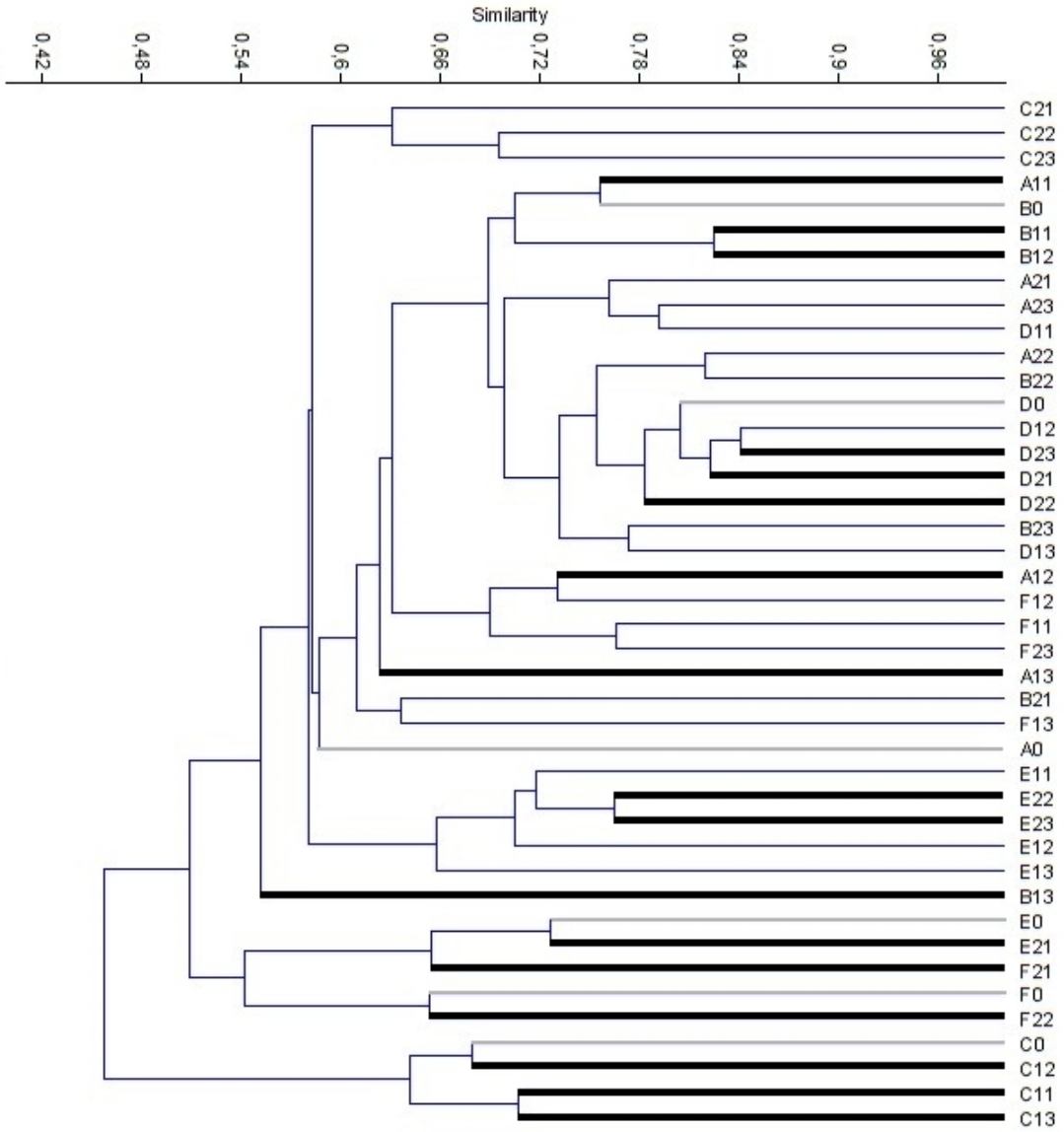

Figure 2. Bray Curtis similarity tree based on T-RFLP profiles from faecal samples. Letters A- F indicate different horses, first number indicates testing period and second number week in period. Thick lines indicate samples from horses with peat as bedding material and thin lines indicates samples from horses with wood shavings as bedding material.

Dry matter and water holding capacity of bedding materials

The water holding capacity of peat was significantly ($p<0.05$) higher than wood shavings when using method A (table 1). There was not a significant difference between periods or samples within periods. There was however a tendency ($p<0.1$) of a difference between periods.

There was a tendency of a difference ($p<0.1$) in water absorption between materials when using method B.

Table 1. Water holing capacity of peat and wood shavings

	Period 1		Period 2	
	at start up	after 3 weeks	at start up	after 3 weeks
Method A				
Peat	33.4 %	34.7 %	37.5 %	39.5 %
Wood shavings	16.5 %	16 %	19.5 %	16 %
Method B				
Peat	43 %	53 %	56 %	31 %
Wood shavings	24.5 %	28 %	29 %	24 %

The dry matter of wood shavings was significantly ($p<0.5$) higher than the dry matter of peat (table 2). The changes in dry matter were not significant.

Table 2. Dry matter of bedding materials

	Period 1		Period 2	
	at start up	after 3 weeks	at start up	after 3 weeks
Peat	43 %	53 %	42.7 %	62.2 %
Wood shavings	88.2 %	84.6 %	88.4 %	83.5 %

pH of bedding materials

Peat had a significantly ($p<0.05$) lower pH-value than wood shavings. The pH had increased significantly in the end of the periods. The pH-value of peat ranged from 3.70 to 3.75 in the beginning of the periods. After three weeks had passed, the pH had increased and ranged from 3.86 to 4.19. The wood shavings had a pH-value of 5.17 to 5.21 in the beginning of the test periods which then increased to 6.40 to 6.45 in the end of the periods.

Microbiological quality of bedding materials

The water activity was significantly higher ($p<0.05$) in peat than in wood shavings. The water activity of peat was always >0.96 while the water activity of wood shavings ranged from 0.62 in the beginning of period 1 to 0.80 in the end of the period.

The same moulds that were present initially were found in peat after three weeks in the first period (table 3). *Penicillium* spp. was always the most common mould when cultivated at 25 °C on DG18 agar. *Paecilomyces* spp. was the most common mould when cultivated in 37 °C on CzapekDox agar. In the second period, yeast and mould were detected which had not been found initially.

Table 3. Moulds, yeasts and fungi present in the Sphagnum peat samples.

	Period 1		Period 2	
	at start up	after 3 weeks	at start up	after 3 weeks
Dominating mould composition, 25 °C	*Penicillium* spp.	*Penicillium* spp.	*Penicillium* spp.	*Penicillium* spp.
Dominating mould composition, 37 °C	*Paecilomyces* spp.	*Paecilomyces* spp.	*Paecilomyces* spp.	*Paecilomyces* spp.
Moulds, direct cultivation, 25 °C	*Penicillium* spp.	*Penicillium* spp.	*Penicillium* spp.	*Penicillium* spp. Yeasts
Thermophilic fungi, direct cultivation, 37 °C	*Paecilomyces* spp. *A. fumigatus*	*Paecilomyces* spp. *A. fumigatus*	*Paecilomyces* spp. *A. fumigatus*	*Paecilomyces* spp. *A. fumigatus* Other moulds

The species of moulds found in wood shavings were not as consistent as the ones found in peat (table 4). The dominating composition of moulds differed between the two periods and moulds found initially were not always present after 3 weeks and vice versa. *Penicillium* spp. was however found as one of the most common moulds in both bedding materials.

Table 4. Moulds, yeasts and fungi present in the wood shavings samples.

	Period 1		Period 2	
	at start up	after 3 weeks	at start up	after 3 weeks
Dominating mould composition, 25 °C	Other moulds	*Penicillium* spp.		*Penicillium* spp.
Dominating mould composition, 37 °C		*Paecilomyces* spp.	*A. fumigatus*	*A. fumigatus*
Moulds, direct cultivation, 25 °C	*Penicillium* spp. *Eurotium* spp.	*Penicillium* spp. *Cladosporium* spp. Yeasts	*Penicillium* spp. *Eurotium* spp. Zygomycetes, Other moulds	*Penicillium* spp. Other moulds *Cladosporium* spp. Yeasts
Thermophilic fungi, direct cultivation, 37 °C	Other moulds	*Paecilomyces* spp. *A. fumigatus* *Zygomycetes*	*Paecilomyces* spp.	*Paecilomyces* spp. *A. fumigatus* Other moulds

There were no significant differences between peat and wood shavings with regard to number of aerobic bacteria, yeasts, moulds and thermophilic fungi (table 5). There was however a significant increase ($p<0.05$) in the number of aerobic bacteria in both bedding materials.

Table 5. Number of aerobic bacteria, yeast, mould and thermophilic fungi in the bedding materials (cfu/g).

			Aerobic bacteria	Yeast	Mould	Thermophilic fungi, direct cultivation, 37 °C
Peat	Period 1	at start up	log 5.8	log 7.6	log 3.3	>log 4.0
		after 3 weeks	log 7.8	log 7.3	log 6.4	>log 4.0
	Period 2	at start up	log 6.9	log 6.8	log 6.3	>log 4.0
		after 3 weeks	log 8.5	>log 7.5	log 6.8	>log 4.0
Wood Shavings	Period 1	at start up	log 5.2	<log 2.0	log 3.3	<log 2.0
		after 3 weeks	log 8	log 5	log 4.5	log 3.3
	Period 2	at start up	log 4	<log 3.0	<log 3.0	log 2
		after 3 weeks	log 7.5	log 4.1	log 4.2	log 2.3

Hygienic quality of haylage

The pH-value of the collected samples from the first period was 6.1 and from the second period 5.2 (table 6). Yeasts and clostridium spores were found to be below log 2 cfu/g in both periods. The composition of moulds and fungi differed from period 1 to period 2.

Table 6. Hygienic quality of haylage in period 1 and 2.

	Period 1	Period 2
pH	6.1	5.2
Yeast (cfu/g)	< log 2	< log 2.0
Mould (cfu/g)	log 3.4	log 2.8
Dominating mould composition, 25 °C	Other moulds	Other moulds
Enterobacteriacae (cfu/g)	log 4.1	log 3.6
Clostridium spores (cfu/g)	< log 2.0	< log 2.0
Moulds, direct cultivation, 25 °C	*Eurotium* spp. *Penicillium* spp.	*Aspergillus* spp. *Penicillium* spp. Other moulds *Eurotium* spp.
Thermophilic fungi, direct cultivation, 37 °C	*A. fumigatus*	Other moulds

Discussion

Nor the faecal pH or the T-RFLP analysis shows any indication that the bedding material would have any impact on the horses' microflora. In the T-RFLP analysis, only horse C had samples that clearly clustered together in separated groups for each period i.e. treatment. Most of the horses have samples that cluster together. One exception was horse A that showed large variation between samples collected in the two experimental periods. Both horse A and horse C are considered to have a large appetite making it not unlikely for them to have consumed some of their bedding. The horses were fed roughage directly on top of their bedding which could be considered a normal exposure to the bedding material.

In similarity with the results from the study by Airaksinen *et al*. (2006), wood shavings had less capacity of water absorption compared to peat. *Penicillium* spp. was the most common fungi found in peat which agrees with earlier results by Larsson *et al.* (1999) and Airaksinen *et al*. (2001). Contrary to results by Airaksinen *et al*. (2006), there were no significant differences between peat and wood shavings considering aerobic bacteria, yeasts, moulds or thermophilic fungi. Poor hygienic quality of the bedding material may cause respiratory disease and possibly digestive disturbances (Frape, 2004). Many *Penicillium* species produce mycotoxins which have various health effects upon ingestion or inhalation. Mycotoxins can cause acute toxicity or chronic health disorders (Adams *et al.* 1993). Mycotoxins are known to negatively affect reproductive performance, cause liver and kidney damage, reduce growth rate, cause colic and death (Frape, 2004; Adams *et al.* 1993). Spores from *A. fumigatus* may cause respiratory allergies (Frape, 2004).

Wood shavings gained water in the stable environment, giving it lower percentage of dry matter and less absorption capacity (method A) whereas peat emitted water and got higher dry matter and absorption capacity after three weeks.

During a three week period there was only a significant increase in the number of aerobic bacteria. The same bedding can however be kept for a much longer time and in order to find out how the quality would change over a longer time span, such an experiment must be conducted.

Even though the horses were being fed the same diet indoors throughout the trial, influence on the microflora can not be excluded due to availability to pasture when being outside. The study was conducted during spring which gave the horses some access to fresh pasture.

Conclusions

With an exposure to the bedding materials such as under these conditions there appears to be no effect on the microflora.

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