Sveriges lantbruksuniversitet
Fakulteten för veterinärmedicin och husdjursvetenskap

Swedish University of Agricultural Sciences
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Molecular characterization of the faecal fungal flora in healthy horses

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Examensarbete / SLU, Institutionen för husdjurens utfodring och vård, **421**

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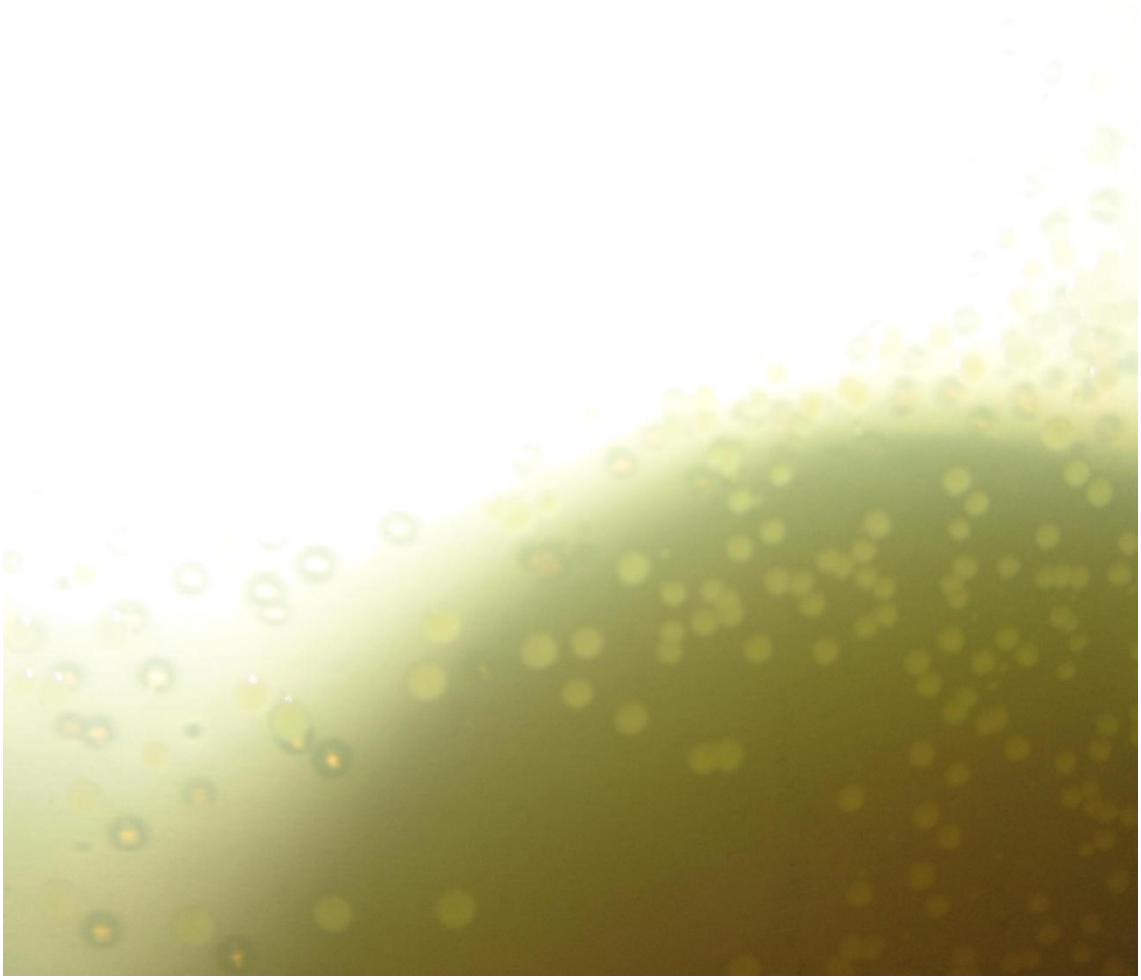

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Abstract

There is a lack of knowledge on the composition and dynamics of the intestinal micro-eukaryotes of horse and to date their functional role for the health and physiology of the host has been described merely to some extent. Particularly, no culture-independent surveys of the dynamics of the intestinal fungal flora in horse are available. Two culture-independent approaches, terminal restriction fragment length polymorphism (T-RFLP) and cloning & sequencing, were combined to study the composition and dynamics of the intestinal fungal flora in healthy horses. The fungal internal transcribed regions (ITS) and the eukaryotic 18S rRNA genes, recovered from faecal samples of 16 Swedish Standardbred geldings, were used as phylogenetic marker genes in the analyses. Faecal samples were analyzed by T-RFLP and two clone libraries were constructed, one library for the 18S gene and the other for the ITS gene from which later 44 random clones were sequenced. T-RFLP profiles and sequencing data together, indicate that the fungal community is temporally stable, has a low diversity and it is dominated by three genera of the *Neocallimastigaceae* family including *Orpinomyces* sp., *Anaeromyces* sp. and *Piromyces* species. These fungal species have been isolated and characterized previously in different types of herbivores including the *Equidae* family. These anaerobic fungi have been suggested to exist prevalently in the GI tract of large herbivores which in this aspect, there is no disparity between the data presented in this experiment and those from previous publications. T-RFLP, as a high-throughput culture-independent method which so far has been implemented mainly on the studies targeting the prokaryotic members of the microbiota, is suitable for the survey of the faecal fungal flora of horses, however requires further adaptations.

Introduction

It is widely believed that the intestinal microbiota has a crucial role for gastrointestinal (GI) functioning and host health via the development and maintenance of host immunology, physiology and nutrition (Scanlan & Marchesi, 2008). This microbial complex plays a vital role in the establishment of the immune system and performs as a barrier against pathogenic threats. Recent research has highlighted that shifts in this ecosystem is accompanied with several human diseases, including inflammatory bowel disease, atopic disorders and obesity (Handl *et al*., 2011). In horses, some forms of colic and laminitis allegedly are due to the alteration in microbial ecology and fermentation pattern in the hindgut of this species (Willing *et al.*, 2009).

Microorganisms in the equine gut maintain the functionality and stability of the GI tract in case of facing environmental stresses and instabilities (Sadet-Bourgeteau & Julliand, 2010). The microbial population in the gut is also of great concern and importance for the host nutrition. These microorganisms are able to digest the structural carbohydrates of feeds that cannot be degraded by intrinsic enzymes. Microbial fermentation of these carbohydrates generates short chain fatty acids that can be utilized by the host animal and emphasizes the importance of the gut microbiota for nutrient utilization (Uden & Van Soest, 1982).

Since the first report by Sprengel (1832) that acetic acid and butyric acid were produced in the rumen as the result of degradation of plant materials, the fundamental function of gut microbes has been rapidly appreciated. Thereafter, protozoa and bacteria were identified in the digestive tract of cattle and horses as major inhabitants (Teunissen & Op den Camp, 1992). It is well established that there are no vertebrates capable of producing cellulases and/or hemicellulases, while few invertebrates are able to do so. However, through the evolution, herbivores have evolved a symbiotic relationship with bacteria, protozoa and fungi. These microorganisms produce and secrete the aforementioned enzymes, causing the evolved herbivores able to exploit plant polymers (Trinci *et al.*, 1994; Hausner *et al.*, 2000).

Equine gut microbiota consists of fungi, protozoa, viruses, archaea and bacteria. These microorganisms are distributed in several segments of the equine alimentary tract. Bacteria have been found in all segments of the GI tract in high concentrations; however, viruses, protozoa and fungi have been reported to be present only in the hindgut (Sadet-Bourgeteau & Julliand, 2010).

In a fed animal, the bacterial load in the large intestine varies between 5×10^8 to 5×10^9 bacteria / g content (Frape, 2010). The fungal load is considerably lower and ranges from 2×10^2 to $2{,}5 \times 10^3$ fungal units/g content. The protozoa are present in about 5×10^4 to 1.5×10^5/ml contents. Together with bacteria and fungi, protozoa participate in the degradation of pectin and hemicellulose in the equine large intestine (Frape, 2010). However, protozoa do not seem to have a significant role in fermentation of feedstuff in the equine hindgut. In fact, bacteria and anaerobic fungi are suggested as the major microorganisms responsible for the degradation of plant tissues in herbivores since defaunation (removal of protozoa) resulted only to a slight reduction in dry matter digestibility with no effect on cellulose digestibility (Moore &

Dehority, 1993). In addition, animals that went through defaunation seem to remain healthy (Trinci *et al.*, 1994).

One study based on metagenomic analysis of the microbiota unravelled that viruses outnumber the other microorganisms in equine faeces, bacteria are the second major group and archaea together with eukaryotes (protozoa and fungi) are the less dominant microbial species (63% of sequenced clones related to viruses, 20% to bacteria, 7% to archaea and 6% to eukaryotes) (Cann *et al.*, 2004). These results are in accordance with those obtained from culture dependent studies, confirming that viruses and bacteria are the most numerous microorganisms, while eukaryotes are fewer (Sadet-Bourgeteau & Julliand, 2010).

It is believed that anaerobic fungi are ubiquitous eukaryotes in the digestive tract of ruminants and in the hindgut of fermenting herbivores. Moreover, since these microorganisms have been isolated in different geographic origins, it seems that anaerobic fungi are distributed globally (Trinci *et al.*, 1994). The anaerobic chytrid fungi in rumen or hindgut of herbivores exploit feed fibre by colonizing plant material and secreting effective fibrolytic enzymes. Type of diet affects the composition of gut microbiota and it has been reported that a high fibre intake increases the fungal population (Nicholson *et al.*, 2010). Amongst herbivores, horses and the African elephant possess a main fermentation in the hindgut. Huge numbers of anaerobic fungi were identified colonizing plant fragments in fresh faecal samples obtained of these mammals (Bauchop, 1981).

In 1975, accidentally, Orpin discovered the first GI anaerobic fungi *Neocallimastix frontalis,* while trying to isolate anaerobic ciliate protozoa from the sheep rumen. Earlier studies though, described *Neocallimastix frontalis* flagellates as flagellate protozoa (Liggenstoffer *et al.*, 2010). Hitherto, six genera of anaerobic fungi and 20 species have been identified and characterized in the GI tract of large herbivores (Griffith *et al.*, 2009; Liggenstoffer *et al.*, 2010). These include the genera *Neocallimastix*, *Piromyces*, *Orpinomyces*, *Anaeromyces*, *Caecomyces* and *Cyllamyces* (Nicholson *et al.*, 2010). All anaerobic fungi so far discovered, have an essential characteristic that is their ability to produce and secrete polysaccharide degrading enzymes including cellulases, xylanases and glucosidehydrolases. For instance, *Neocallimastix frontalis,* has been shown to hold an effective cellulolytic activity (Teunissen & Op den Camp, 1992).

Traditionally, recognition and morphological classification of gut fungi have been based on cultivation methods or on the microscopic observation of zoospores of anaerobic fungi in the gut content (Liggenstoffer *et al.*, 2010; Nicholson *et al.*, 2010). Results from culture based and molecular studies have been compared and it appeared that large numbers of microorganisms yet cannot be cultured in the lab. Particularly when it comes to anaerobic habitats, problems associated with culture dependent studies tend to get aggravated, since anaerobic microorganisms are intolerant of exposure to oxygen that deters mycologists to study anaerobic types (Lin *et al.*, 1997). Anaerobic fungi tend to show substantial variation when being cultured on different substrates or under different growing conditions. Moreover, isolation and maintenance of anaerobic fungi is troublesome and subsequent to a long time of being cultured, most of the polycentric species would not produce or develop mature sporangia which make identification of older species difficult (Hausner *et al.*, 2000).

The limitation of culture based approaches has driven the research towards the application of molecular techniques. The progress of molecular techniques allowed for a more thorough identification of microbial communities by detecting temporal and spatial changes in microbial structure (Nicholson *et al.*, 2010).

Recently, a number of molecular methods have been developed for the assessment of microbial communities which are referred as microbial community fingerprinting methods (Dicksved, 2008). Examples of such techniques are cloning & sequencing, temperature gradient gel electrophoresis (TGGE), denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP). The common characteristics of these methods is that they are based on the PCR amplification of either the small sub-unit rDNA (18S for yeasts and fungal species and 16S for bacterial species) or the internal transcribed spacer (ITS) region for fungal members (Lord *et al.*, 2002).

The 18S rRNA gene and the internal transcribed region (ITS) gene are the most prevalent molecular markers in phylogenetic studies and biodiversity screening of fungi (Gardes & Bruns, 1993). The rRNA genes encoding 16S, 5.8S and 28S, are conserved to extreme level and hence in studies of *Neocallimastigales* members, implementation of the ITS region has shown to be successful (Nicholson *et al.*, 2010). Furthermore, due to the lower level of conservation, the ITS region has been suggested to be more accurate for classification of the anaerobic gut fungi compared with the 18S rRNA gene, which is highly conserved (Brookman *et al.*, 2000).

Amongst the microbial fingerprinting methods, T-RFLP is one of the most popular and has been carried out in analyses dealing with bacterial 16S rRNA genes, fungal ribosomal genes and archaeal 16S rRNA genes (Schutte *et al.*, 2008). PCR amplification of the ribosomal small sub-unit genes from community extracted DNA is the initial step in T-RFLP analysis. In this step, one or both of the conserved primers are end-labelled with a fluorescent dye (Schutte *et al*., 2008). In the subsequent step, restriction digestion of the amplicons with one or several enzymes is performed followed by a separation of fragments with gel electrophoresis (Schutte *et al.*, 2008; Lord *et al.*, 2002). During the gel electrophoresis the DNA fragments passes a fluorescence detector that will record the intensity and sizes of the fluorescent labelled fragments. Different steps of T-RFLP analysis are illustrated in Figure 1.

Compared to DDGE and TGGE, which are both gel-based techniques, T-RFLP has a higher throughput due to application of an automated DNA sequencer technology (Anderson & Cairney, 2004; Lord *et al*., 2002). Furthermore, in studies of complex microbial communities in natural habitats like GI tracts or soils, T-RFLP method is verified to have better resolution than other PCR based fingerprinting approaches (Kitts, 2001).

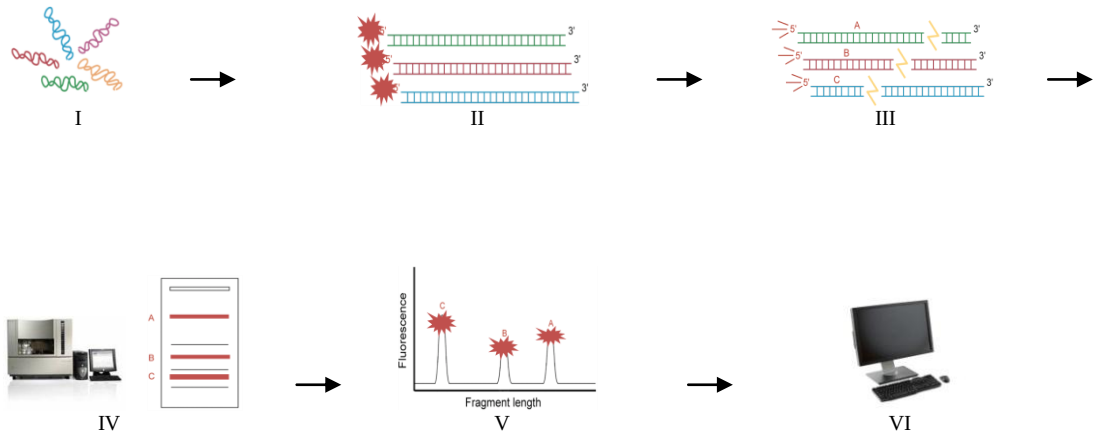

Figure 1. (I) DNA extraction, (II) PCR amplification with fluorescently end-labelled primers, (III) Restriction digestion of the amplicons, (IV) Separation and detection, (V) Visualization by software, (VI) Data analysis.

The first culture independent study of the fungal community inhabiting the mammalian GI tract was carried out by Scupham *et al.* (2006). They used molecular tools to explore the diversity and abundance of fungi populating the murine GI tract. Their study identified a fungal community pertaining to nine different taxonomic clusters and that fungi constitute around 2% of the total microbiota of the caecal biofilms (Scupham *et al.* 2006).

The initial step of understanding how host factors and nutrition are influencing the micro-eukaryotic population of the GI tract, is to identify the diversity and to quantify the abundance of microorganisms from digesta or faecal samples. By virtue of fistulated animals, direct access to microbial populations of the GI tract has been feasible. This approach enables temporal and comparative examinations of the gut microorganisms (Nicholson *et al.*, 2010). In addition, studying the influence of different parameters such as the environmental alterations or changes in diet and how these parameters influence the microbial population has been facilitated via fistulation (Sadet-Bourgeteau & Julliand, 2010). Nevertheless, for large-scale evaluations and comparisons of many herbivores, fistulation is not appropriate. Faecal samples on the other hand are easily accessible and sampling does not require surgical and invasive procedure. While many studies have been done on faecal samples, still the drawback is that samples may not represent GI tract microbiota, neither quantitatively or qualitatively (Sadet-Bourgeteau & Julliand, 2010).

There have been advances in the knowledge of the microbial ecology inhabiting different segments of the equine GI tract. Despite those advances, a lack of knowledge about microorganisms is yet considerable (Sadet-Bourgeteau & Julliand, 2010). This is particularly evident for the micro eukaryotic microbiota. Thus, the initial aim of this study was to identify the fungal species inhabiting the equine GI tract and to scan the dynamics of this eukaryotic community over time using the T-RFLP method.

The composition and dynamics of the gut micro eukaryotes so far have been characterized in human and some mammals using molecular approaches, whereas considerably few studies have targeted the equine species and horses and those few

studies have focused mainly on the structure and classifications of the anaerobic fungi and paid less attention to the dynamics of the gut eukaryotes which accordingly makes this study unique. Furthermore, to our knowledge this is the first study using the T-RFLP method to target micro eukaryotes in the horse gut.

Materials and Methods

Sample collection and preparation

16 Swedish Standardbred gelding were included in the experiment. All horses were born in the spring of 2009 and were kept in the National Centre for Trotting Education in Wången, Sweden. The horses were kept individually in boxes and were fed on forage-only diet which mainly consisted of haylage and silage. All horses were healthy during the experiment and they did not show any sign of specific disease. Moreover, they were kept under good condition from an animal welfare point of view. In fact, the horses were part of a larger study which focused on different physiological and locomotive aspects of the Swedish trotter horses when their diet contained only forage. Samples were picked shortly after defecation and placed in plastic bags. Each bag was labelled with date and the corresponding horse's name and shortly after labelling, bags were placed into a freezer set at -20 °C. The first sampling was done in October 2010 and the last one was carried out in October 2011. In total, 9 sampling occasions were included in the experiment. In order to obtain a representative sample, drill cores from three parts of each faecal sample were collected using a 12mm diameter drill (Pluggborr, CraftOMAT, Germany). Afterwards, these cores were inserted into a 50 ml plastic tube and placed into a freezer set at -20 °C for further procedures.

In addition to faecal samples, two feed samples were obtained from two different haylage batches and placed in a freezer set at -20 °C. One feed sample was picked from a haylage fed to horses in November 2010 (#1) and the other one was taken from a haylage fed during May 2011 (#5).

DNA extraction

DNA extraction was carried out according to the protocol for isolation of DNA from stool for pathogen detection, within QIAamp DNA Stool Handbook (QIAGEN, Germany), with some minor modifications. Prior to each DNA extraction, the tubes containing faecal samples were taken out from the freezer and 5 ml of phosphate buffered saline (PBS) was added to each tube. Samples were thawed and homogenized using a vortex. In the first step of the procedure, 180 to 220 mg of thawed faecal sample was placed into a 2 ml screw cap tube containing 400 mg of 0.5 mm glass beads (BioSpec products). The vortex step in the second step of the protocol was replaced by a bead beating step, 2 x 45 seconds at full speed using a mini beadbeater (BioSpec products). This replacement was done to increase the rupture of fungal tissue. The following steps were performed according to the manufacturer's instructions. In the last step, 1.5 ml microcentrifuge tubes containing extracted DNA were labelled with the corresponding horse's name and placed into a freezer set at -20 °C for subsequent processes.

In addition to faecal samples, DNA was isolated from two feed samples using the same protocol as for faecal samples, however with some initial preparatory steps.

Prior to DNA extraction, 15 g of each sample was weighed and inserted into standard bags for the Stomacher 400 circulator (Seward, United Kingdom) and mixed with 150 ml of PBS. The samples were then homogenized in the Stomacher for 2 minutes at a speed of 230 rpm. Afterwards, the liquid phase of the sample was collected and centrifuged at 5000 rpm for 5 minutes to pellet small particles. The supernatant fluid was removed and approximately 500 µl of precipitate was placed into 2 ml screw cap tube containing 400 mg of 0.5 mm glass beads. The protocol for DNA extraction was performed in the same way as for the faecal samples with one exception, in the second step of the procedure, 1.2 ml of the ASL buffer was added to each 2 ml screw cap tube instead of adding 1.4 ml of ASL buffer as outlined in the protocol. At the end, 1.5 ml tubes containing extracted DNA from feed samples were labelled and stored in -20 °C until further analyses.

Nucleic acid concentration was measured using the BioSpec-nano instrument (Shimadzu, USA) on random samples (24 samples in total). DNA yields varied to a large extent amongst samples, while nucleic acid concentration about 7.0 ng/µl was appeared to occur often.

PCR amplification

Two different eukaryotic genes were amplified in the study. First, the fungal internal transcribed region (ITS) gene was amplified using the primers ITS1F-Fam 5'-CTTGGTCATTTAGAGGAAGTAA-3') (Gardes & Bruns, 1993) and ITS4R (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990). ITS1F was fluorescently labelled with Fam (Carboxyfluorescein) at the 5' end of the primer.

The final volume of each PCR reaction was 20 µl and consisted of 0.5 µM of each primer, 2 µl of DNA templates, 0.5 µg/µl of bovine serum albumin (Fermentas, Germany) and DreamTaq PCR Master Mix (2X) according to the manufacturer's instructions (Fermentas, Germany). The PCR cycles consisted of an initial heating step at 94 °C for 3 minutes, followed by 30 cycles of denaturation at 94°C for 40 seconds, annealing at 50 °C for 40 seconds and extension at 72 °C for 60 seconds. The PCR was finalized with a final extension step at 72 °C for 5 minutes. DNA extracted from 48 faecal samples was PCR amplified by ITS primers. These faecal samples were taken from 9 different sampling months and 14 horses were involved in this schema. Table 1 is illustrating the faecal samples that were included in this step of the analysis.

Table 1. Samples selected for analysis with ITS primers.

Horse name	Oct. 2010	Nov.2010	Dec. 2010	Jan. 2011	Mar. 2011	May. 2011	Jun. & Jul. 2011	Aug. 2011	Oct. 2011
Period	1	2	3	4	5	6	7	8	9
Staro Gimli		X		X					
Be Mine	X						X	X	
Fabian Palema		X	X		X				
Adams Tor	X				X	X	X	X	
Windy Palema		X		X		X&X			
Mordor Broline				X	X				
Staro Gretzky	X	X		X					
Staro Gin Tonic (Groggen)	X	X			X	X			

Staro Golden Eye								X	X
Staro Glory Days	X&X				X	X	X		
Robert Palema	X			X		X			X
Hjalmar Palema		X		X		X		X	
Lasken Palema							X	X	
Mon Cheri Broline	X		X			X	X	X	

The eukaryotic genes encoding 18S rRNA were PCR amplified using the primers Euk1A-Fam (5'-CTGGTTGATCCTGCCAG-3') and Euk516r (5'-ACCAGACTTGCCCTCC-3'). The forward primer (Euk1A) was labelled with a fluorescent dye (Fam) at the 5' end. The 18S genes were amplified using the same PCR chemistry as for ITS, and the PCR amplification was carried out with an initial heating at 94 °C for 3 minutes, followed by 35 cycles of denaturation at 94 °C for 40 seconds, annealing at 55 °C for 40 seconds, elongation at 72 °C for 60 seconds and ended with a final elongation for 7 minutes at 72 °C. Table 2 displays the faecal samples that were included in amplification of 18S genes.

Table 2. Samples selected for analysis with 18S primers.

Horse name	**Abbreviation**	**Oct. 2010**	**Nov. 2010**	**Jun. & Jul. 2011**	**Aug. 2011**
Period		1	2	7	8
Staro Gimli	GI	X		X	
Sunpower H. P.	SP		X		X
Be Mine	BM		X	X	
Fabian Palema	FP		X	X	
Adams Tor	AT	X		X	
Windy Palema	WP	X		X	
Mordor Broline	MB	X			X
Staro Gretzky	GR	X		X	
Staro Gin Tonic (Groggen)	GT	X		X	
Staro Golden Eye	GE	X			X
Staro Glory Days	GD	X		X	
Robert Palema	RP	X		X	
Hjalmar Palema	HP	X			X
Lasken Palema	LP	X		X	
Mon Cheri Broline	MC	X		X	
Staro Gipsy King	GK	X		X	

Following each PCR reaction, aliquots of PCR products (5 µl) were analysed with gel electrophoresis to confirm that PCR products of expected size were obtained. All faecal and feed samples were PCR amplified in duplicates. Moreover, in order to avoid any chance of contaminations in each individual PCR reaction, a negative sample was included. This negative sample contained nuclease-free water instead of DNA templates and was capable of detecting contaminations in each PCR run.

T-RFLP analysis of PCR products

The amplicons (in duplicates) from the previous step were digested with endonucleases to produce DNA fragments with variable length. Aliquots (10 µl) of amplified ITS genes were digested separately at 37 °C for 2 hours with FastDigest TaqI in a mixture according to the manufacturer's instructions (Fermentas, Germany). 18S PCR products were digested separately with two endonucleases. Aliquots (10 µl) of the 18S rRNA amplified genes were individually digested for 2 hours at 37 °C with FastDigest AluI or FastDigest HaeIII according to instructions by the manufacturer (Fermentas, Germany).

Following digestion, the fragments were separated on a polyacrylamide gel with an ABI automated sequencer 3730 (Applied Biosystems, USA). In the electrophoresis, the DNA fragments passed a fluorescent detector that measured the intensity of the end-labelled fragments and determined the length of fluorescently labelled fragments using an internal standard. Subsequently, electropherograms of the TRFs were analysed and visualized by Peak scanner software version 1.0 (Applied Biosystems, USA). In the data analysis, all fragments with sizes below 50bp were excluded, to remove fluorescent signals from mono- or di-mers from the PCR primers. The data from analyzed samples were subsequently exported to Microsoft Excel (2007) software for further data treatments.

T-RFLP data treatments

The first step of the data process was to calculate the relative abundance of each TRF. This was done by dividing the peak area for each TRF with the total peak area for all TRFs in the sample. In the next step, a lower threshold level was set at a relative abundance 0.5% and all TRFs with a relative abundance lower than 0.5% was excluded from further analyses. The PCR replicates were then adjusted so different TRFs of each duplicate were compared to each other and based on comparison between their sizes, TRFs were deleted wherever there was not a pair of TRFs with equal sizes in both replicates. The subsequent step was to merge duplicates into one consensus TRF profile. This step was done by calculating the average peak area for each TRF in the duplicate. Thereafter, sizes were rounded to remove decimals and the merged samples were sorted according to different sizes. Next, the data were exported to Microsoft Access (2007) to create a final matrix that included sample names, TRF sizes and their relative abundance. The data matrices of ITS amplicons and 18S amplicons are available as appendix.

Clone library construction and analysis

Two clone libraries were constructed in this experiment; the first one was based on ITS amplicons and the other from 18S PCR amplified genes. Duplicate PCR products were generated from 6 horses (FP23, GT62, MC13, HP63, GI43 and GD63)) using primers ITS1F and ITS4R (both unlabelled) by the same PCR protocol outlined previously for ITS primers. Subsequently, duplicates were pooled and PCR product sizes confirmed with gel electrophoresis. The PCR products were excised from the agarose and extracted from the gel using QIAquick gel extraction kit and according to the manufacturer's protocol (QIAGEN, Germany). Following the gel extraction, samples were randomly divided into two pools, each containing three samples.

Afterwards, the two amplicon pools were transformed into chemically competent *Escherichia coli* using TOPO TA Cloning kit (Invitrogen, Sweden) according to the manufacturer's instructions. One of the libraries established successfully (HP63, GI43 and GD63) and the other one failed.

The clone libraries of eukaryotic 18S genes was constructed as per the protocol that was outlined for ITS. Similar to the ITS library setup, the initial plan was to establish two clone libraries from 18S PCR amplified genes. Four random faecal samples, from four different horses (AT13, HP13, RP73 and MC73), were selected for this purpose. However, only one of the libraries established successfully (from samples HP13 and MC73), the other failed in the end.

The next step was sequencing analysis and prior to that, in order to match TRF size with sequence data, T-RFLP analysis was done on random clones from the clone libraries. The objective was to pick clones that would belong to different eukaryotic species, in other words, to avoid sequencing similar species. This comparison of TRFs between samples and clones has been done successfully in previous research, for instance it was outlined by Lindahl *et al.* (2007). Therefore, from the ITS clone library, a total of 47 random clones were analyzed by T-RFLP. Selection was based on TRFs data assessment and scanning TRFs peaks by Peak scanner software. From the 18S clone library, 48 random clones were analyzed by T-RFLP and the same selection process as for the ITS clone library was implemented, except that clones were analyzed by T-RFLP separately with two endonucleases, HaeIII and AluI.

The same process as for faecal samples was carried out in T-RFLP analysis of clones, except that small amounts of colony material was used as template in the PCR. The PCR cycles consisted of initial heating at 94 °C for 3 minutes, followed by 30 cycles of denaturation at 94°C for 40 seconds, annealing at 55 °C for 40 seconds, extension at 72 °C for 60 seconds and a final extension at 72 °C for 5 minutes. The PCR reaction for the 18S clones library contained the primers Euk1A-Fam and Euk516r and ITS1F-Fam and ITS4R were used as primers in the PCR reaction of clones from the ITS clone library.

Followed by T-RFLP assessment, 24 clones from the ITS library were chosen for sequencing and from the 18S library, 20 clones were selected. Subsequently, the chosen clones were PCR re-amplified using primers directed towards the TOPO vector (M13f and M13r) and identical PCR chemistry and program as for the T-RFLP analysis of the clones. The generated products were sent for sequencing to a sequencing facility (Macrogen Inc, The Netherlands).

In total, 44 sequences were obtained and all of them were analysed by the Finch TV software (version 1.4.0). Finch TV was used to identify vector regions of the sequence. The proper 18S or ITS sequence were selected and matched against the GenBank database using standard nucleotide BLAST at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). BLAST is a web-based algorithm that can be used to match an obtained sequence with sequences deposited to the public databases. Using BLAST, it was possible to identify the corresponding species of individual sequences.

Results

Optimization of T-RFLP protocol for fungal analysis

In this experiment, T-RFLP combined with cloning and sequencing of genes encoding 18S rRNA or the ITS region, were used to assess the structure and stability of the equine micro-eukaryotic microbiota. As indicated earlier, there was no publication using T-RFLP to study the fungal species in the horse gut and from the beginning of the experiment it appeared that there is a need to optimize the method. There were two main goals in optimization of the method; first to strengthen the amplicons and then selecting the appropriate endonuclease. At the beginning of T-RFLP analysis, PCR products were happened to be weak, specifically ITS amplicons. To enhance this situation, some modifications were done like changing the annealing temperature (from 55 °C to 50 °C for ITS gene), altering PCR cycles (from 30 cycles to 35 for ITS gene) and increasing the DNA templates intensity or that of primers. However, each modification improved the weakness of amplicons very slightly and none of them had a significant effect. As the second step of the method modification, some experiments were carried out to select the most effective enzymes amongst available endonucleases for both ITS and 18S amplicons. In fact, there are some publications in which fungal species have been targeted, however in other natural habitats like compost, soil, tree etc. Consequently, those methodologies were taken into consideration and the enzymes that have been used previously, were tested in this experiment and assisted us in this step to choose the proper endonucleases. For ITS amplicons, selection was made between AluI, HaeIII and TaqI. At first, DNA, extracted from 12 random faecal samples was PCR amplified and then digested separately with the aforementioned enzymes. The digested amplicons were separated by electrophoresis and accordingly, TaqI was selected as a suitable endonuclease for the digestion process, since this enzyme appeared to cleave amplicons at more sites than the others. The gel picture from electrophoresis is illustrated in Figure 2. This picture shows that HaeIII was virtually ineffective in cleavage of amplicons, while AluI digested amplicons to some extent, yet the cleavage was less than TaqI.

For 18S amplicons, a similar process was performed to choose the most suitable endonuclease. DNA extracted from 6 random faecal samples in addition to 2 feed samples were PCR amplified and then digested separately with AluI, HaeIII and TaqI. Subsequently, electrophoresis showed that all enzymes has digested amplicons, however, AluI and HaeIII were more effective than TaqI, since their restriction pattern were more complex compared with the restriction pattern generated by TaqI. Figure 3 displays three representative samples from the aforementioned electrophoresis.

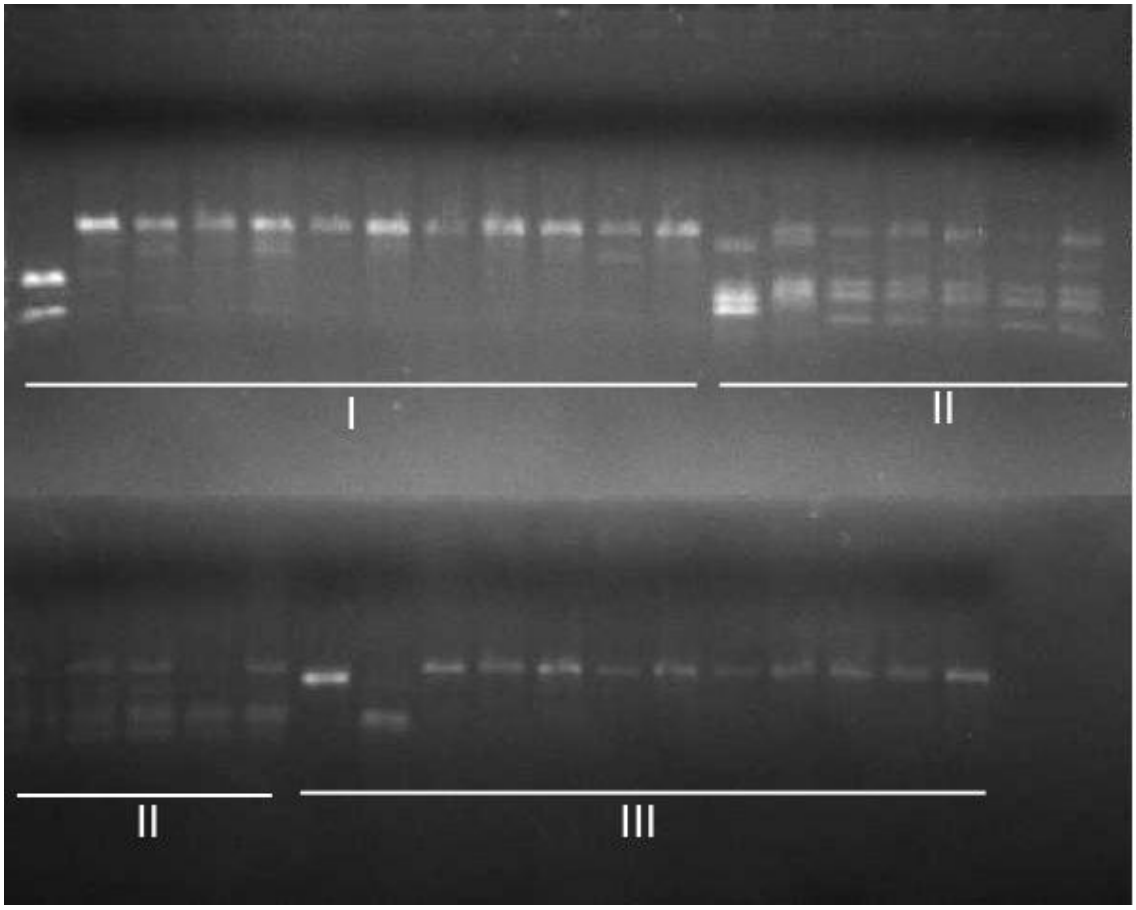

Figure 2. Electrophoresis after digestion of 12 samples separately by AluI (marked as I), TaqI (marked as II) and HaeIII (marked as III), respectively.

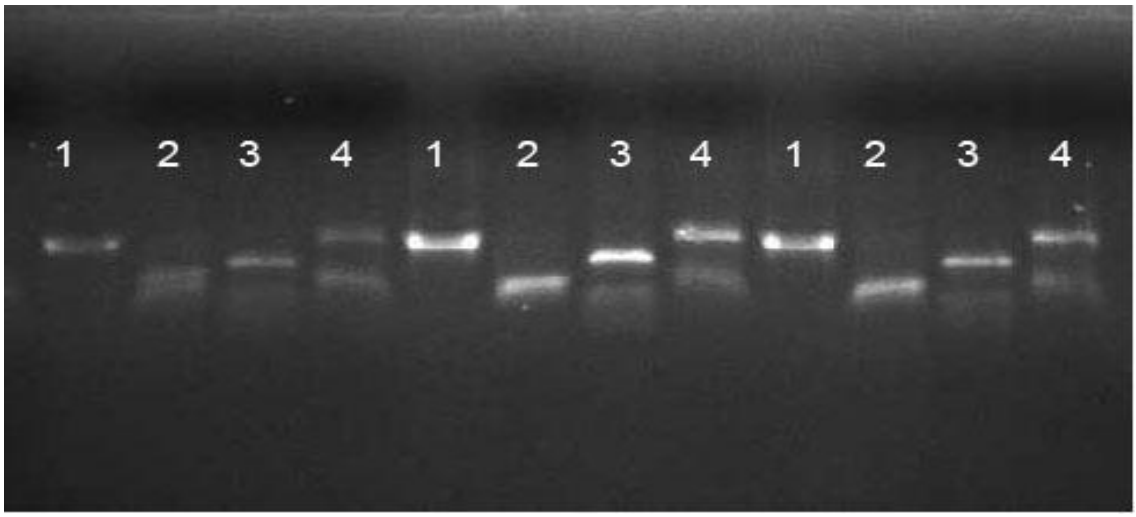

Figure 3. Electrophoresis after digestion of 3 different samples separately by HaeIII (marked as 2), AluI (number 3) and TaqI (number 4), respectively. Number 1 represents undigested DNA bands, each of them belongs to different samples.

T-RFLP analysis on ITS amplicons

In total, T-RFLP data was obtained from 48 faecal samples using the ITS primers. T-RFLP data was visualized by the Peak scanner software and the obtained electropherograms were studied and compared. Obtained data from the scanner software were exported to Microsoft Excel and after being treated, the final matrix was acquired. The ITS graphs showed few peaks in the majority of the samples, indicating a microbial community with few members. Peaks were also quite stable within an individual sample over a period of time. Two random selected graphs are illustrated in Figures 4 and 5. As can be seen, these graphs belong to two different horses and even two different season, one of them (RP13) was taken in October and the other (GT62) was from May, however both graphs have three peaks in the same regions, only with slightly different intensity. The ITS matrix displayed four regions of peak clusters, the first of them located at peak size of 86, the next one between 187-193 peak sizes, then among 377-380 and the last one was between 386-388.

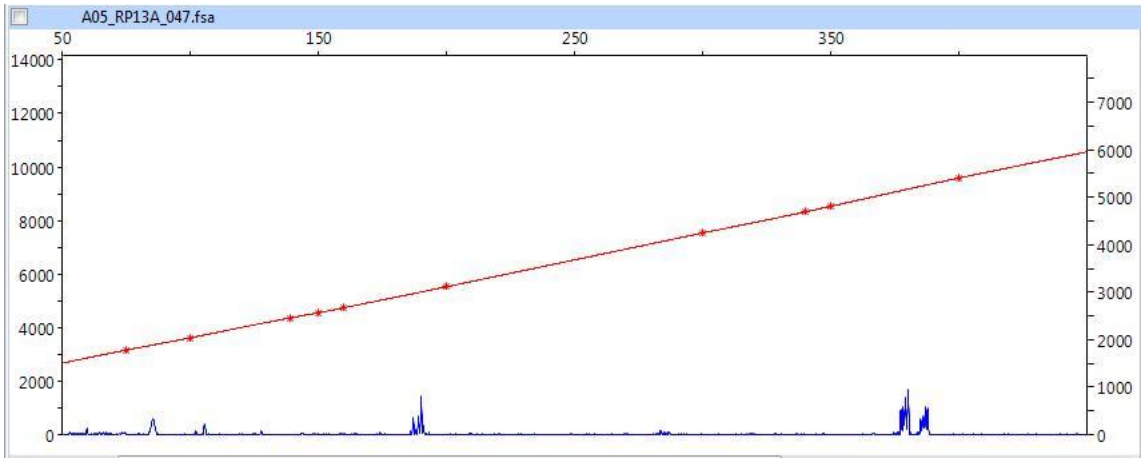

Figure 4. Electropherogram obtained from a faecal sample RP13 digested by TaqI.

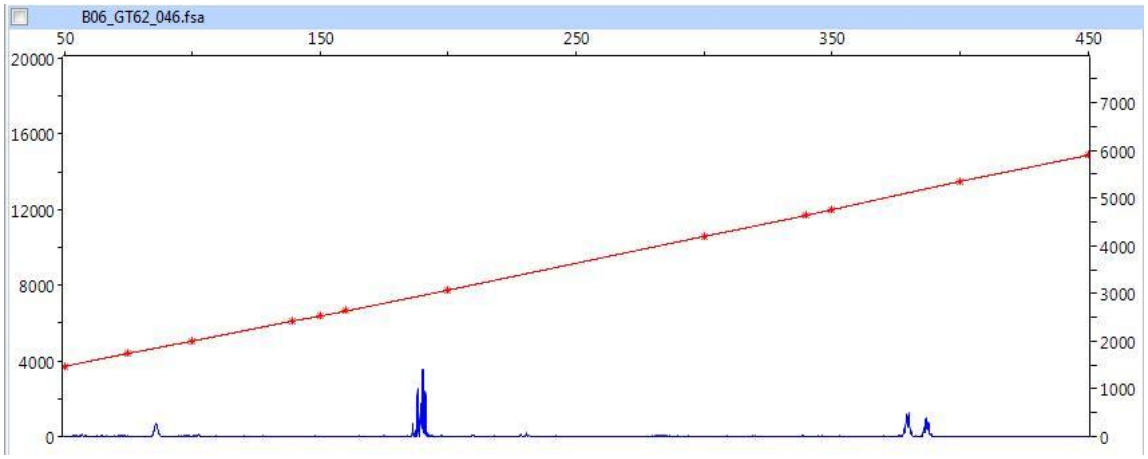

Figure 5. Electropherogram obtained from a faecal sample GT62 digested by TaqI.

T-RFLP analysis on 18S amplicons

34 samples (including 32 faecal samples plus 2 feed samples) were PCR amplified by 18S primers and thereafter analyzed by T-RFLP. The obtained data was visualized by the Peak scanner software and also two matrices was created by

Microsoft Excel after data treatment, one belonged to the samples digested by HaeIII and the other one belonged to those digested by AluI. The 18S electropherograms displayed a simple microbial community with few peaks. Scanning samples peaks showed that the fungal population was virtually stable over time; in other words, there were no significant seasonal changes in the community dynamics.

Figures 6 and 7 illustrate two electropherograms obtained from two faecal samples which were taken in different seasons, one in October and the other in June & July, respectively. There were more peak cluster in the 18S matrices compared to the ITS matrix. In addition, matrix of samples digested by HaeIII has slightly more regions of peak clusters than the other matrix belonged to AluI digestion.

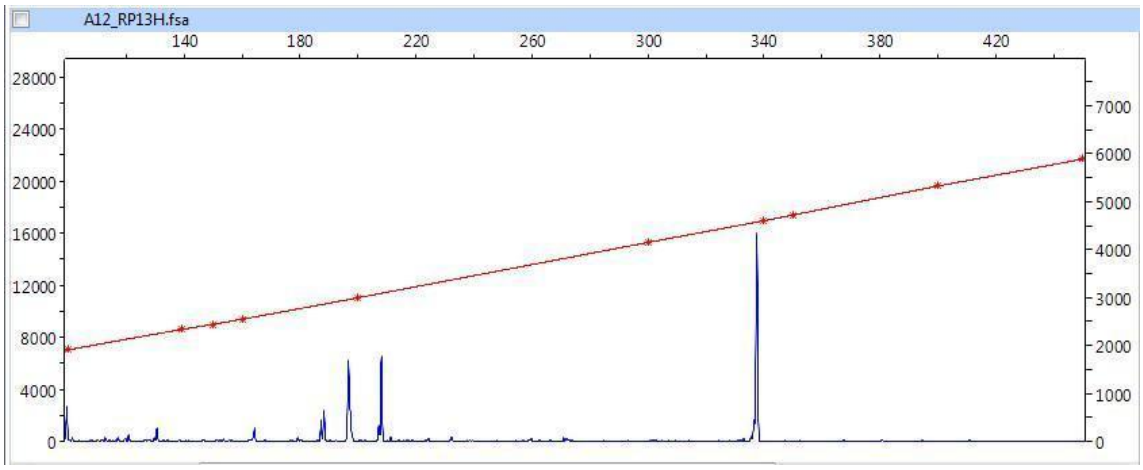

Figure 6. Electropherogram obtained from a faecal sample RP13 digested by HaeIII.

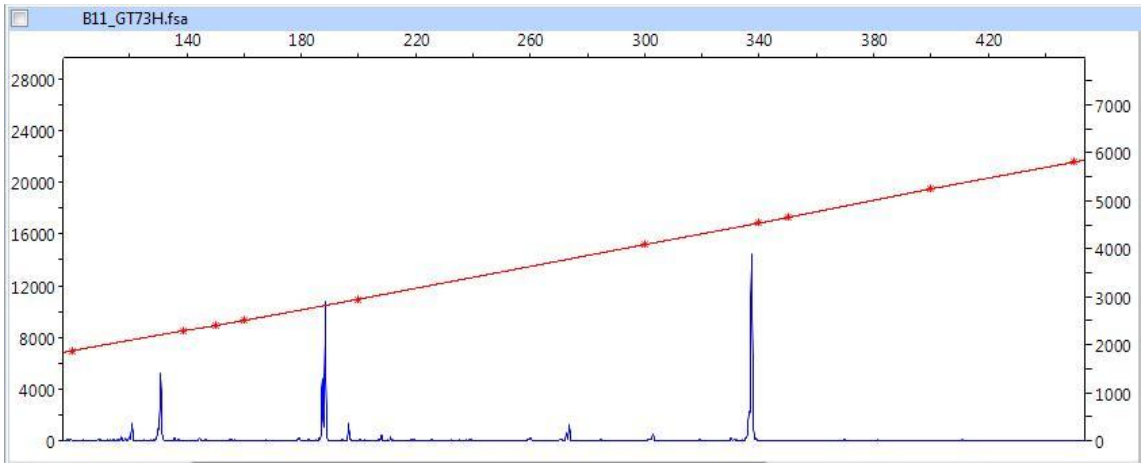

Figure 7. Electropherogram obtained from a faecal sample GT73 digested by HaeIII.

Cloning and sequencing

The next part of the experiment was to create clone libraries in order to identify the fungal species residing in the GI tract of horses. ITS and 18S amplicons were cloned into vectors and sequenced as outlined earlier. In total, 44 sequences were obtained in this experiment, 24 of them were from the ITS clone library and 20 from the 18S clone library. Prior to sequencing, all of those 44 clones were analyzed by T-RFLP. The TRFs obtained from ITS and 18S clones were compared with TRFs from faecal

samples and it was shown that highest peaks of both clones exist in faecal samples, too. All the sequences were matched against the public data bases using the BLAST algorithm to identify the most probable identity of the species. This procedure was performed for all sequences.

ITS sequencing result

Table 3 illustrates the sequencing results from the ITS clone library and it contains sample label, the highest peak size in the electropherogram, best database match and maximum identity score. The ITS sequencing analysis shows that most of the species are anaerobic fungi and belongs to two genera of the *Neocallimastigaceae* family, *Anaeromyces* and *Piromyces*. There are, however, two exceptions which are *Geomyces* and *Debaryomyces hansenii* (samples EK1 and EK2, respectively) which both belong to *Dikarya* subkingdom and *Ascomycota* phylum. Moreover, the maximum identity score of these two species is 99% that is higher than those of other samples belonging to the *Anaeromyces, Piromyces* and *Orpinomyces* genera (the highest score is 86%). Furthermore, the highest peak size of samples EK1 and EK2 are different from those of other samples (respectively, 334 and 362). Most of samples belong to *Neocallimastigaceae* family have a peak size at 190 or 378 with few exceptions, however, none of them has a peak size similar to the first two samples. These peak sizes (190 and 378) are within the range of the four peak clusters of the ITS matrix that is outlined earlier. This verifies the fact that anaerobic fungi can be found in most of the faecal samples in this experiment. Since all of the participating horses in this experiment were Swedish Standardbred, it is likely that anaerobic fungi exist in the GI tract of at least this special breed, even if not all the other horse breeds.

Table 3. Sequencing results from ITS clone library.

Samples label	Highest peak size in electropherogram	BLAST first hit	Max identity
EK1	334	Geomyces sp.	99%
EK2	362	Debaryomyces hansenii strain	99%
EK3	190	Anaeromyces sp.	85%
EK4	190	Anaeromyces sp.	85%
EK5	190	Anaeromyces sp.	85%
EK6	192	Piromyces sp.	85%
EK7	189	Piromyces sp.	85%
EK8	387	Piromyces sp.	86%
EK9	380	Anaeromyces sp.	85%
EK10	381	Anaeromyces sp.	85%
EK11	187	Piromyces sp.	86%
EK12	189	Anaeromyces sp.	85%
EK13	190	Piromyces sp.	86%
EK14	190	Anaeromyces sp.	85%
EK15	190	Anaeromyces sp.	85%
EK16	189	Piromyces sp.	81%
EK17	187	Anaeromyces sp.	86%
EK18	384	Anaeromyces sp.	85%
EK19	378	Piromyces sp.	85%
EK20	378	Anaeromyces sp.	85%

EK21	378	Piromyces sp.	86%
EK22	379	Orpinomyces sp.	83%
EK23	379	Anaeromyces sp.	86%
EK24	386	Piromyces sp.	85%

18S sequencing result

The sequencing results from the 18S library are illustrated in Table 4. The only detected fungal species was *Orpinomyces* which is a genus that belongs to the *Neocallimastigaceae* family. One difference compared with the sequences generated from the ITS library was that the sequences generated from the 18S library had a higher maximum identity score (99% similarity) compared to those obtained from the ITS library sequencing. The peak size of *Orphinomyces* was 161 for TRFs obtained by AluI and 336-337 for TRFs acquired by the HaeIII enzyme. Similar to the ITS matrix, the peak sizes representing anaerobic fungi can be found in peak clusters of the 18S matrices. Almost all of the faecal samples have a peak size of 161 with the AluI enzyme and most of the faecal samples have a peak size of 336 or 337 when HaeIII was used. This shows that this fungal family is a permanent member of the eukaryotic microorganisms of the horse gut, at least in this special breed of horse. Sequence analysis also classified one sequence as best match to the intestinal parasite *Entamoeba.* The pivotal issue from 18S amplicon library was that there were some species that belonged to the *Plantae* kingdom, like *Lolium multiflorum* or *Secale cereale*. In fact, this phenomenon was entirely unexpected and it is regarded as the major drawback of this part of the experiment. It can indicate that either the PCR protocol or primer sequences were not discriminative enough to exclude plant DNA.

Table 4. Sequencing results from18S clone library.

Samples label	Highest peak size (AluI)	Highest peak size (HaeIII)	BLAST first hit	Max identity
EK25	161	336	Orpinomyces sp.	99%
EK26	159	271	Secale cereale	99%
EK27	160	40	Secale cereale	99%
EK28	159	270	Lolium multiflorum	99%
EK29	161	337	Orpinomyces sp.	99%
EK30	160	271	Secale cereale	99%
EK31	161	336	Orpinomyces sp.	99%
EK32	161	337	Orpinomyces sp.	99%
EK33	160	273	Secale cereale	99%
EK34	160	271	Secale cereale	98%
EK35	161	336	Orpinomyces sp.	99%
EK36	161	336	Orpinomyces sp.	99%
EK37	156	197	Entamoeba sp.	87%
EK38	160	37	Lolium multiflorum	100%
EK39	159	271	Secale cereale	99%
EK40	160	271	Secale cereale	99%
EK41	161	336	Orpinomyces sp.	99%
EK42	159	271	Lolium multiflorum	99%

EK43	160	40	Secale cereale	99%
EK44	160	271	Lolium multiflorum	96%

Discussion

Similar to the other farm animals and even humans, little is known about the diversity, dynamics and function of eukaryotic microorganisms inhabiting the equine GI tract (Scanlan & Marchesi, 2008; Handl *et al.*, 2011). The primary goal of this experiment was to examine the microbial ecology of micro-eukaryotic organisms in the equine gut using the culture independent method T-RFLP. In addition, identification of congruency of results obtained from the classical methods and culture independent methods was another aim of this experiment.

One noticeable problem in this experiment was that PCRs from both ITS and 18S primers generated weaker PCR products than expected beforehand (especially ITS amplicons were weak). Several trials were done to optimize the PCR method, however the attempts to optimize the PCR methods for both ITS region and 18S genes were not successful and the PCR products were in general weak. This could be due to several reasons, for instance, incidence of PCR inhibitors in extracted DNA samples or inappropriate design of the utilized primers. Apart from mentioned reasons, this could also be due to the shortage of fungal gene in faecal samples. Previous research by Cann *et al.* (2004) revealed that in faecal samples of horses, only 6% of the sequenced microbial genes belonged to eukaryotes which show the scarcity of micro-eukaryotic genes in faecal samples of horses. Although it has not been verified whether this weakness in the PCR step has affected the other downstream applications in the experiment or not, it is regarded as a potential source that might have influenced T-RFLP and sequencing results.

T-RFLP peaks being visualized by the Peak scanner software indicate that the diversity of eukaryotic microorganisms in the equine faecal samples is low (e.g. most of the samples have between two and three peaks). Moreover, TRF peaks show that the dynamic of the eukaryotes is somewhat stable over time. Faecal samples analyzed for ITS regions were obtained randomly from different time period (Table 1) and shows a stable banding pattern over time within each horse (Figures 4 and 5). In the analysis using 18S amplicons, faecal samples were acquired from two time periods (winter 2010 and summer 2011; Table 2) and the T-RFLP results confirm this stability over time. Data analysis of the TRF pattern shows a similarity in TRF profiles between different samples. Similarity between TRFs of different horses or those belong to one individual taken from different periods of time, implies the stability and low diversity of the gut eukaryotes at least in this special breed of horse. Results from cloning and sequencing imply that the genera from the *Neocallimastigaceae* family are the most common eukaryotes in the equine faecal samples and presumably in the equine GI tract, while this has been proven by the both approaches i.e. 18S and ITS.

The ITS data process led to a matrix in which most of the samples have their peaks in four separate zones of peak size which was outlined earlier. T-RFLP analysis of clone libraries revealed that most of the TRFs sizes from the clones lies within these

four size ranges, except for three clones which are EK1, EK2 and EK18 (Table 3). Both clone EK1 (TRF size 334) and clone EK2 (TRF size 362) had unique TRF sizes and these were the only identified clones with sequences that not belonged to the *Neocalligastigaceae* family. This indicates that a TRF pattern with sizes within the above mentioned zones (sizes 86, 187-193, 377-380 and 386-388bp) could be used to identify members of the *Neocalligastigaceae* family.

In the study, 91.6% of the obtained sequences were matched against sequences identified as anaerobic fungi that belonged to three genera: *Piromyces*, *Anaeromyces* and *Orpinomyces*. These three genera all belongs to the *Neocalligastigaceae* family. According to the highest peak size criterion, the identified *Piromyces sp*. can be divided into three distinct TRF groups; the first group had TRF sizes between 187-192bp, the second group around 378bp and the last one between 386-387bp (Table 3). These different TRFs pattern for one genus may represent different species within the genus *Piromyces*. So far, using classical methods, researchers were able to isolate and identify three distinct species in equine including *Piromyces mae* (Li *et al*., 1989)*, Piromyces rhizinflata* (Breton *et al*., 1991) and *Piromyces citronii* (Gaillard-Martinie *et al*., 1995). It can however be seen from Table 3, that TRFs belonging to *Anaeromyces* sp. in some cases have the same size as those for *Piromyces sp*., for instance in samples EK7 and EK12. This implies that T-RFLP analysis for ITS region did not distinguish between different genera within the *Neocalligastigaceae* family, at least not for all analysed samples. The identified *Anaeromyces* genus can be divided into two groups, one of them have peak sizes in a range of 187-190 and the other one have peak sizes of about 380 (between 378-384), meanwhile each of these groups could represent a distinct species of this genus (Table 3). In fact, so far, there are no reports of the isolation and identification of the *Anaeromyces* species from the horse gut. However, two species have been isolated and described from ruminants using classical methods; *Anaeromyces elegans* and *Anaeromyces mucronatus* (Trinci *et al.*, 1994). In the analysis of sequences generated from ITS amplicons, a low max identity scores for fungal species (around 85%) were obtained (Table 3). One reason for that could be that the number of sequences in the databases is limited for this type of microorganism, another reason could be that these are novel species.

The 18S data process led to two matrices that showed that most of the samples were dominated by few TRF sizes. Identification of the TRFs by cloning and sequencing revealed that *Orpinomyces* sp. was detected as the only type of fungal species. To date, two species of *Orpinomyces* have been isolated and identified from the rumen; *Orpinomyces bovis* from cattle and *Orpinomyces joyonii* from sheep (Teunissen & Op den Camp, 1992). In addition, one unknown species of *Entamoeba* genus was detected by 18S rRNA analysis. *Entamoeba* genus has several species which are parasites residing in the intestinal lumen of human and animals, some of them are pathogenic e.g. *Entamoeba histolytica*, while some of them are non-pathogenic (Fotedar *et al*., 2007). It can be seen that the highest peak of *Entamoeba* sp. has a TRF size of 197 with HaeIII enzyme and its analogous size is 156 produced by AluI endonuclease (Table 4). These peaks are obviously different from those of other species which supports the proper functioning of 18S rRNA analysis. It can be seen from 18S matrices (HaeIII and AluI) that most of faecal samples have a peak around these sizes i.e. 156 and 197, except for feed samples which do not possess such

peaks. This verifies *Entamoeba* genus does not exist in the feed samples which is a positive item for feed hygiene.

To date, studies using culture dependent methods have isolated and described few anaerobic fungal species from horses and ponies. *Piromyces citronii* was isolated from the caecum of both pony and donkey (Gaillard-Martinie *et al*., 1995), *Piromyces mae* has been isolated from faeces derived from the hindgut of a horse (Li *et al*., 1989) and *Caecomyces equi* was isolated from the horse caecum and its vegetative and reproductive stages were described (Gold *et al.*, 1988).

The first culture independent survey of anaerobic fungi in faecal samples from a wide range of herbivores (10 different families) used the 454 pyrosequencing method and was done by Liggenstoffer *et al.* in 2010. They found that most of the sequences in samples from the *Equidae* family belonged to novel anaerobic fungi and the other sequences were affiliated to four genera of anaerobic fungi including *Neocallimastix*, *Piromyces*, *Caecomyces* and *Anaeromyces*. According to their report, feed type, gut type and animal host phylogeny are the factors in determining the anaerobic fungal diversity and community composition in different hosts. However, animal host phylogeny seems to be the most important factor in this aspect. Moreover, it is important to notice that by October of 2009, only 236 ITS sequences of anaerobic fungi are available in GenBank which indicates that the current knowledge of the diversity of anaerobic fungi is little (Liggenstoffer *et al.*, 2010).

A number of studies have been successfully utilized T-RFLP method to investigate the fungal community dynamics and/or compositions in different habitats, for instance in soil (Dickie *et al.*, 2002); marsh (Buchan *et al.*, 2002); wood (Allmer *et al.*, 2006); Camembert cheese (Arteau *et al.*, 2010); boreal forest (Lindahl *et al.*, 2007); manure compost (Tiquia, 2005) etc. Perhaps, one of the closest research to this experiment is the one performed by Tiquia in 2005. Tiquia scanned the dynamic of the fungal population and the bacterial communities in a manure compost which was consisted of equally one portion of cow manure and one portion of horse manure. The author used EF3 and EF4 primers for targeting the 18S gene and also utilized HhaI, RsaI and MspI as endonulcleases. Due to the application of different T-RFLP protocols, precise comparison of our data (i.e. data from Tiquia study and those of ours) is impeded. Yet, based on diversity indices and visual observation of the TRFs in this study, it was concluded that the fungal population have a lower diversity than the bacterial population in manure in the initial phase of composting and that T-RFLP showed to have a high potential for monitoring microbial changes in compost.

It is obvious from previous studies and the results from this experiment that *Neocallimastigaceae* family members are prevalent in the GI tract of herbivorous animals which implies a large capacity for these species to survive in the GI tract of various hosts and hence indicates a versatile genome. Scupham *et al.* (2006) studied the fungal population in the murine intestine using a culture independent method and reported that *Neocallimastix* genus was amongst the prevalent fungal genera of the murine GI tract. Therefore, anaerobic fungi are not limited only to the herbivores and can be found in non-herbivores as well.

In construction of the ITS clone library, only three random faecal samples from different horses were used (HP63, GI43 and GD63), even two of those samples were from a same sampling occasion. In the end, only 24 clones were sequenced. Similarly for the 18S clone library, only two faecal samples were chosen (HP13 and MC73) and finally 20 of the established clones were subjected to sequencing analysis. Collectively, samples from few horses were utilized for cloning and subsequently few clones were sequenced. The later was due to the financial matters. It would be desirable and more comprehensive to use faecal samples from all horses and from different seasons for cloning and furthermore, sequencing a large number of clones. This would result in a more comprehensive dataset that could promote a more robust conclusion. However, due to limitations in the project budget, this dataset was not possible to obtain.

This study has dealt substantially with the methodological aspect. There was a comparison between 18S and ITS primers and collectively it turned out that different approaches have their advantages and disadvantages. The 18S approach was more suitable than the ITS approach in the aspect that it led to stronger PCR products and higher resolution to distinguish between different species in T-RFLP analysis. Although, the main drawback was the unspecific detection that allowed amplification of several plant species in the PCR. A more optimized PCR protocol or modifications in the primer sequence could solve this issue. One potential advantage with the ITS protocol was that the protocol was more selective and identified more fungal species than the 18S protocol. It is probable that T-RFLP analysis has distinguished between different species in this experiment, the accuracy and correctness of this assumption could however not be determined.

Liggenstoffer *et al.* (2010) have described the desirable form of studies on anaerobic fungi and reasoned that only an optimized study can lead to reliable results. Accordingly, a well-controlled study on replicates from members of only a single animal species with different age, diet and geographical regions can provide precise information on community dynamics and structure and factors affecting these criteria. This study has followed these criteria and in a small scale shed light on the fungal communities dynamics and composition in the GI of horses fed on forage-only diet; however, further studies are required to get a deeper coverage and understanding of the intestinal fungi of horses and this can be achieved using T-RFLP and/or in combination with other sequencing approaches.

Nowadays, there are small group of researchers, though dedicated, that are studying anaerobic fungi (Liggenstoffer *et al.*, 2010). However, little is known about the function of GI fungal community in GI health and disease and further detailed investigation of the GI fungal role is necessary (Handl *et al.*, 2011).

Conclusions

The results of our study suggest that the diversity of the fungal population inhabiting the guts of the hosts is low and most of the species belong to *Neocallimastigaceae* family which are anaerobic fungi. Previous studies have identified anaerobic fungi in the digestive tract of different herbivores (ruminants and non-ruminants) including

horse. Likewise, our results verify that anaerobic fungi are the dominant micro-eukaryotes in the horse GI tract. T-RFLP method carried out in this experiment was not able to differentiate distinctive microorganisms at the *species* level, but only at *family* level. Considering the importance of the fungal flora for physiology and health status of host and the obvious lack of knowledge in this research field, one can realize the urge of having more research in this area. Future studies may try to vary the conditions of participating horses e.g. involving different breeds of horse (including warmbloods and coldbloods) and pony, feeding different type of diets etc., since this might lead to more comprehensive results and finally a thorough and certain conclusion for all types of horses.

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