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Anaerobic fungal communities differ along the horse digestive tract

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Abstract

Anaerobic fungi are potent fibre degrading microbes in the equine hindgut, yet our understanding of their diversity and community structure is limited to date. In this preliminary work, using a clone library approach we studied the diversity of anaerobic fungi along six segments of the horse hindgut: caecum, right ventral colon (RVC), left ventral colon (LVC), left dorsal colon (LDC), right dorsal colon (RDC) and rectum. Of the 647 ITS1 clones, 61.7% were assigned to genus level groups that are so far without any cultured representatives, and 38.0% were assigned to the cultivated genera *Neocallimastix* (35.1%), *Orpinomyces* (2.3%), and *Anaeromyces* (0.6%). AL1 dominated the group of uncultured anaerobic fungi, particularly in the RVC (88%) and LDC (97%). Sequences from the LSU clone library analysis of the LDC, however, split into two distinct phylogenetic clusters with

low sequence identity to *Caecomyces* sp. (94-96%) and *Liebetanzomyces* sp. (92%) respectively. Sequences belonging to cultured *Neocallimastix* spp. dominated in LVC (81%) and rectum (75.5%). Quantification of anaerobic fungi showed significantly higher concentrations in RVC and RDC compared to other segments, which influenced the interpretation of the changes in anaerobic fungal diveristy along the horse hindgut. These preliminary findings require further investigation.

Keywords: Anaerobic fungi; Diversity; Uncultured; ITS1; Horse; Hindgut

1. Introduction

Horses evolved as free-ranging herbivores of grassland environments, with an enlarged hindgut adaptation enabling them to obtain energy and nutrients from plant structural polysaccharides through microbial fermentation. The hindgut is comprised of two main fermentative chambers, the caecum and colon, which together constitute two-thirds of the volume of the digestive tract. The hindgut has a combined capacity of over 200 L (Frape 2010) and accounts for 75% of the mean transit time (23-48h) of dietary particles (van Weyenberg et al. 2006). The hindgut is home to bacteria, anaerobic fungi, methanogenic archaea and protozoa. Of these, the anaerobic fungi (Neocallimastigomycetes) are the most potent in terms of degrading plant fibres due to their complete and very efficient set of plant cell-wall degradation enzymes (Gruninger et al. 2014, Haitjema et al. 2014). Despite their presence in the horse hindgut within a few weeks of birth (Julliand et al. 1996), almost all the current knowledge of anaerobic fungi is derived from ruminant based studies.

Nine genera of anaerobic fungi validly described based on cultivated representatives, including those with filamentous monocentric (*Neocallimastix*, *Piromyces*, *Oontomyces* and *Buwchfawromyces*), filamentous polycentric (*Orpinomyces*, *Anaeromyces* and *Pecoramyces*) and bulbous (*Caecomyces* and *Cyllamyces*) mycelium (Gruninger et al. 2014, Edwards et al. 2017), have been recently extended by new monocentric genera *Feramyces* (Hanafy et al. 2018) and *Liebetanzomyces* (Joshi et al. 2018). These genera, however, represent only part of anaerobic fungal diversity as indicated by the large and growing numbers of internal transcribed spacer region 1 (ITS1) sequences in public databases that belong to potentially novel, as yet uncultured clades within the Neocallimastigomycetes. Based on the re-evaluation of publicly available ITS1 sequences, which took into account both primary sequence and secondary structure information, Kittelmann et al. (2012) and later Koetschan

et al. (2014) proposed a revised phylogeny and pragmatic taxonomy of anaerobic fungi, which resulted in 37 reproducible species or genus-level clades. Eighteen of these clades have not been cultured, containing only environmental derived sequences, and the number of not yet cultivated clades was recently increased to twenty-five (Paul et al. 2018).

A survey of anaerobic fungi in faeces of 30 different ruminant and non-ruminant herbivore species, found that members of the family Equidae clustered apart from the other non-Equidae herbivores studied (Liggenstoffer et al. 2010). The different domesticated and non-domesticated equines sampled in the Liggenstoffer et al. (2010) study shared a similar anaerobic fungal community, which was mainly composed of two novel genus level groups that are now termed AL1 & AL3 (Koetschan et al. 2014). Cultivated anaerobic fungal genera were found in equine samples only in limited relative abundance (4-12% of *Caecomyces*, 2% of *Neocallimastix*, 0.3% of *Piromyces*, 0.1-0.3% of *Anaeromyces*). These results suggest that the digestive tract of horses is largely occupied by novel, as yet uncultured anaerobic fungi, which differ from those previously described from foregut herbivores.

Whilst molecular based analysis of equine anaerobic fungal diversity has been performed on faecal samples (Liggenstoffer et al., 2010), it is known that bacterial, protozoal and archaeal community composition differs with hindgut segment (Julliand and Grimm 2016; Fliegerova et al. 2016). Therefore, there is a clear need to assess if the anaerobic fungal community composition also differs along the hindgut, particularly as niche differentiation within the anaerobic fungi has previously been proposed (Griffith et al. 2009). In this preliminary study, the anaerobic fungal community composition along the hindgut of a mature horse was determined using ITS1 based clone libraries.

2. Materials and Methods

2.1. Collection of digesta samples from the horse hindgut

Gut content samples were taken from the six segments of the hindgut of an Anglo-Arabian gelded male (24 years old) euthanised for non-research purposes in a local abattoir. The horse was maintained on a mixed diet of grass, meadow hay and complementary feed as described by Fliegerova et al. (2016), and was healthy with no history of any intestinal disorders. The segments sampled were: caecum, right ventral colon (RVC), left ventral colon (LVC), left dorsal colon (LDC), right dorsal colon (RDC) and rectum (representing faeces). Gut segments were tied off to prevent mixing between neighbouring segments, and the whole

content from a section mixed thoroughly before a sample (approx 500 g) was taken. Samples were placed in labelled plastic containers and transported on wet ice back to the laboratory where they were frozen (at -20°C) and then freeze-dried. Freeze-dried material was stored at -20°C until DNA extraction.

2.2. DNA extraction

Freeze-dried gut content (5 g) was homogenized using a mortar and pestle with liquid nitrogen. Genomic DNA was then extracted from 400 mg of the resulting powder using the cetyltrimethylammonium bromide extraction protocol of Gardes and Bruns (1993) after modification with the respect to the amount of sample material used. The concentration and purity of extracted nucleic acids was checked using a NanoDrop 2000c UV-Vis spectrophotometer (Thermo Scientific, U.S.A), and DNA extracts were stored at -20°C until use.

2.3. PCR amplification

Amplification of the anaerobic fungal ITS1 region from each sample was carried out with the combination of the fungal universal ITS1 forward primer (Gardes and Bruns 1993) and the Neocallimastigomycetes specific 5.8S rRNA gene reverse primer (Edwards et al. 2008). This resulted in an amplicon of approximately 350 bp in length, as described previously by Fliegerova et al. (2010). The PCR reaction (50 µl) was performed with a PPP Master Mix kit (Top-Bio, Czech Republic) and 0.3 µM of each primer using the following thermal cycling conditions: 33 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 30 s and extension at 72°C for 45 s with an initial cycle of 94°C for 4 min and final cycle of 72°C for 2 min. Each sample was PCR amplified in quadruplicate and then pooled after successful amplification and verified by agarose gel electrophoresis.

For one sample, the LDC, the ribosomal large subunit 28S rRNA (LSU) was also amplified in quadruplicate with universal primers NL1 and NL4 as described previously by Fliegerova et al. (2006). The rationale for this analysis is explained in the results section.

Pooled PCR amplicons of the correct length were excised from an agarose gel with a sterile scalpel blade, purified and concentrated using a QIAquick Gel Extraction Kit (Qiagen, Germany).

2.4. Construction of clone libraries and sequencing

The TOPO® TA Cloning® Kit for Sequencing (Life Technologies, USA) was used for the preparation of the ITS1 clone libraries for each segment of equine hindgut, and the LSU clone library of the LDC segment. Ligation of the PCR amplicons into pCR4-TOPO vector was performed at room temperature for 30 min followed by transformation into competent One Shot TOP10 *Escherichia coli* cells (30 min on ice). Randomly selected clones of *E. coli* grown overnight in lysogeny broth plates with ampicillin (50 µg/ml) were checked by PCR with M13 primers for the presence of the ITS1 and LSU fragments of expected length. Plasmid DNA was then isolated from 881 ITS1 clones and 20 LSU clones using a GenElute™ HP Plasmid Miniprep Kit (Sigma-Aldrich, USA). Purified plasmid DNAs were quantified using a NanoDrop ND-1000 UV-Vis Sectrophotometer (NanoDrop Technologies, USA) and Sanger sequenced (SEQme, Czech Republic). Generated sequences were checked for errors and any vector contamination removed. Of the clones sequenced, 647 of the ITS1 clones and 17 of the LSU clones yielded clear and unambiguous sequence data that were used for further analysis.

2.5. Taxonomic and phylogenetic analysis

Taxonomic assignment of ITS1 sequences (97% identity threshold) was done in QIIME version 1.8 (Caporaso et al. 2010) by BLAST against the anaerobic fungal ITS1 reference database (Koetschan et al. 2014). The 647 cloned sequences with taxonomic assignment from the six libraries were concatenated into a single fasta file, and a sequence map (seqs_otus.txt) was manually built for input into the script "make_otu_table.py". The resulting relative abundance table was summarized at the clade level. A similar process was followed with the LSU sequence data, except the similarity search was carried out using BLAST against GenBank.

Evolutionary analyses of ITS1 sequences and the LSU sequences from the LDC sample were separately performed using the software MEGA 6 (Tamura et al. 2013) to construct phylogenetic trees, with 1,000 replicates for bootstrap analysis, using the UPGMA method. LSU genes of described genera *Caecomyces* (JQ782555, KM878679), *Cyllamyces* (DQ273829, KY386297), *Piromyces* (JF974096, JF974119, JN939159), *Buwchfawromyces* (KP205570, NG058679), *Feramyces* (MG584197, MG584228, MG605676), *Neocallimastix* (JF974094, JN939158, KT274174, KR920745), Pecoramyces (JN939127, KX961618), *Orpinomyces* (HQ703476, JN939163, KM878680), *Anaeromyces* (JN939157, JN939170,

JN939172), *Liebetanzomyces* (MH468763) and *Oontomyces* (JX017314, JX017315) were used as reference sequences.

The ITS1 nucleotide sequences generated from the horse hindgut have been deposited in the GenBank database under the following accession numbers: MH038102 - MH038269 (caecum), MH038399 - MH038497 (RVC), MH038498 - MH038612 (LVC), MH038272 - MH038398 (LDC), MH038613 - MH038694 (RDC) and MH038695 - MH038784 (rectum). The LSU sequences of the LDC have been deposited in the GenBank database under the accession numbers MH125212 - MH125228.

2.6. qPCR analysis

The MX 3005P QPCR System (Stratagene) and the Kapa SYBR Fast qPCR Master mix (Kapa Biosystems) was used for the qPCR determination of ITS1 gene copy numbers in each sample using the ITS1 primer pair and cycling conditions as described above (see PCR amplification). The standard curve was created (in triplicate) using a 10-fold dilution series of a pCR4-TOPO plasmid containing the ITS1 region of a *Piromyces* isolate (NCBI Accession No. KY368107). ITS1 copy numbers were determined (in triplicate) from 1 μl of ten-fold diluted DNA, and data expressed per g of dry matter of digesta. Three replicates of a negative control sample without DNA template were also included in the qPCR assay. The qPCR assay efficiency was 101.5%, and quantitation was linear over eight orders of magnitude (10^1 to 10^8 gene copy μl^{-1}). The unpaired t-test (Microsoft Excel 2010) was used to identify significant differences in the counts of anaerobic fungi among all the samples. Significant differences were declared when $P < 0.05$. A quantitative profile of the anaerobic fungi was then calculated, using the ratio between the total ITS1 gene copy number and relative abundance of each clade for each hindgut segment.

3. Results

3.1. ITS1 based diversity analysis of anaerobic fungi in the horse hindgut

Of the 647 successfully sequenced ITS1 clones from all six segments of the horse hindgut, the 645 clones appeared to be anaerobic fungal in origin and only 2 of these sequences (0.3%) could not be further taxonomically assigned within the class Neocallimastigomycetes using either the anaerobic fungal ITS1 reference database or GenBank. On average 61.7% of all the sequences were represented by genus-level groups that have so far only been described in the literature based on sequence data: AL1, AL7, DT1 and KF1 (as defined by Koetschan

et al. 2014). The cultured genera *Neocallimastix, Orpinomyces* and *Anaeromyces* represented the remaining 38.0% sequences that could be taxonomically assigned. Phylogenetic relationships of ITS1 sequences of uncultured anaerobic fungi generated from the horse hindgut and cultured genera is shown in Fig. S1.

3.2. Distribution and quantification of anaerobic fungi along the horse hindgut

The relative abundance of the different anaerobic fungal clades (as defined by Koetschan et al. 2014) along the six segments of the horse hindgut are shown (Fig. 1.). The AL1 clade represented 53% of the total sequences and was present throughout the hindgut, however, its relative abundance differed among gut segments. AL1 represented almost all of the anaerobic fungi in the RVC (88%) and LDC (97%), and was the most abundant clade in the caecum (62%) and RDC (53%). In contrast, the relative abundance of AL1 in the LVC (4%) and the rectum (3%) was very low. Generally, the decreased abundance of AL1 in certain gut segments seemed to be associated with an increase in the abundance of the *Neocallimastix* 1 clade. This clade was the second most abundant (35.1% of the total sequences) and was detected in all segments. The third most abundant clade was AL7 (4.3% of the total sequences), and was also detected in all segments. In contrast, the KF1 clade (4.2% of the total sequences) was only detected in the LVC (7%), RDC (3%) and rectum (17%). Sequences of the other less abundant fungi (*Orpinomyces* 1a, *Orpinomyces* 1b, *Anaeromyces* 1 and DT1) were relatively minor (13.7% of total sequences) and detected only in some hindgut segments.

Total anaerobic fungal concentrations differed between segments, with the exception of the caecum and LDC (Fig. 2.). The RVC followed by RDC was found to contain the highest concentrations of anaerobic fungi, and the lowest concentrations of anaerobic fungi were detected in the LVC and rectum. It can be clearly seen that the lower concentrations of total anaerobic fungi in these segments is associated with a substantial decrease in the amount of the AL1 clade, rather than an increase in the *Neocallimastix* 1 clade (as indicated by Fig. 1.).

Fig. 1

Fig. 2

3.3. LSU based diversity analysis of the LDC

As 97% of the ITS1 sequences from the LDC segment belonged to the monophyletic AL1 clade (Fig. 3), this segment was also analysed using an LSU based clone library in order to

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417 better elucidate the taxonomic position of this uncultured clade. Tree topology showed that
418 the LSU sequences obtained (n=17) formed two distinct clusters (Fig. 4). The clones
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420 representing cluster I (n=8) had highest sequence identity (94 - 96%) to a sequence belonging
421 to a cultured *Caecomyces communis* (KM878679), and formed a sister group to the
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423 *Caecomyces*/*Cyllamyces* group. The clones representing cluster II (n=9) had highest sequence
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425 identity (91 - 92%) to a sequences belonging to a cultured *Liebetanzomyces* sp. (MH468763)
426 and *Anaeromyces* sp. (MG605690), however, the cloned sequences clustered distantly from
427
428 the reference anaerobic fungi present in the tree.
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430 Fig. 3.
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432 Fig. 4**.**
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435 **4. Discussion**
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437 Gut microbes are essential colonizers of the mammalian digestive tract. They are involved in
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439 food decomposition, production of vitamins and micronutrients. Microbial fermentative end-
440 products (especially acetate, propionate and butyrate) supply the host body by energy.
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442 Microbiota of digestive tract is also involved in a variety of other metabolic and physiological
443 functions including the shaping of the immune system. The importance of the equine
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445 intestinal microbial ecosystem for animal health and performance is well established (Dicks
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447 et al. 2014), however, research has primarily focussed on only bacteria. The investigation of
448 the anaerobic fungi in the equine hindgut to date has been limited and, to the best of our
449
450 knowledge, there is no study of the diversity of anaerobic fungi in the different anatomo-
451 physiological segments of the horse hindgut.
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454 Our work showed that an uncultured *Neocallimastigales* clade named AL1 was prevalent in
455 the equine hindgut. This finding is in agreement with an ITS1 based next generation
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457 sequencing study that found the NG1 (=AL1 in Kittelmann et al. 2012) group could account
458 for 56.7%-99.9% of the total anaerobic fungi in the faeces of certain horses and zebra
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460 (Liggenstoffer et al. 2010). It was surprising to observe that the AL1 clade was greatly
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462 decreased in the LVC and rectum, with the reason for these decreases not clear.
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464 The AL1 clade is not specific to equines, as it was also detected (17.8- 49.5%) in a variety of
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466 other foregut fermenters (Liggenstoffer et al. 2010). However, ITS1 sequences of the AL1
467 clade retrieved in this study were most similar or identical only with those from the zebra and
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469 horse (deposited in the GenBank database). The effort to elucidate the phylogenetic
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relationships of the ITS1 defined AL1 clade using the LSU resulted in splitting of the sequences into two non-related clusters. As this contrasted the monophyletic clade seen with ITS1, further work is needed to confirm the basis of this finding.

The second most numerous clade of uncultivated anaerobic fungi identified in horse faeces by Liggenstoffer et al. (2010), clade NG3 (=AL3 in Kittelmann et al. 2012), has not been found in any part of the horse hindgut sampled in this study. On the other hand, sequences of the group NG7 (=AL7 in Kittelmann et al. 2012) retrieved by Liggenstoffer et al. (2010) in low relative abundance only from Somali wild ass faeces (0.6%), represented 4% of total clones in the present study. Sequences of another group of anaerobic fungi, the KF1 clade, which represented 17% of the sequences in the horse rectum in this study, were first detected in cow manure and were described as an undistinguished *Cyllamyces/Caecomyces* cluster of uncultured fungi (Fliegerova et al. 2010).

Regarding the known cultivable anaerobic fungi, only the clade *Neocallimastix* 1 colonized the hindgut of the studied horse with a dominant relative abundance in the LVC and rectum. However, quantitative profiling showed that this was due to the absence of clade AL1, rather than an increased amount of *Neocallimastix* 1 in these segments. The *Neocallimastix* 1 sequences were mostly similar to those obtained from ruminants, especially from cow, bison and yak. *Neocallimastix* is known by its excellent hydrolytic properties and multi-functional cellulosomal enzymes, and is presumed to be a very effective (hemi)cellulose degrader (Gruninger et al. 2014, Wei et al. 2016). In horse, zebra and donkey faeces analysed by Liggenstoffer et al. (2010) this genus was either absent or represented at very low relative abundance (< 2%). However, in a sample from a Somali wild ass, *Neocallimastix* represented 45% of all sequences.

Whilst the cause of the considerable difference in anaerobic fungal diversity between the RVC and LVC is not clear, the difference between the neighbouring LVC and LDC may be related to the pelvic flexure. This narrow junction is responsible for the selective retention of coarse particles. Less digested particles are retained in the caecum and LVC, whereas liquid and finer particles move on into the LDC. These retropulsive-propulsive movements keep the caecum and the whole ventral colon filled, increasing the fermentation time of plant biomass. These segments of the hindgut are, therefore, the sites with the highest digesta mean retention time of (about 9 hours) and represents the main site of lignocellulose degradation (Van

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535 Weyenberg et al. 2006). As well as a longer time to degrade the structural polysaccharides,
536 this also provides more time for the propagation of microorganisms.
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539 The implication of the RVC ecosystem in forage degradation has been highlighted already by
540 de Fombelle et al. (2003), due to the decreased proportion of cellulose in the digesta after
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542 passing through the RVC. This corresponds well with our finding of highest anaerobic fungal
543 concentrations in the RVC compared to the other hindgut segments. On the other hand, the
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545 segments with the lowest anaerobic fungal concentrations (LVC and rectum) were associated
546 with a decrease in the amount of the AL1 clade. Variations in the concentration of anaerobic
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548 fungi found among different gut segments in this study, however, contrasts with study of
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550 Dougal et al. (2012) where no effect of gut segment was seen based on the caecum, RDC and
551 rectum sites that were sampled.
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554 The RDC is the site of another selective mechanism (known as the colonic separation
555 mechanism). This mechanism is responsible for the prolonged retention of fluid and smaller
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557 food particles (Drogoul et al. 2000). How this mechanism may contribute to the decrease of
558 the AL1 clade in the rectum is not clear. Digesta samples from this part of the hindgut, could
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560 in future studies be separated into a liquid and a solid fraction to provide further insight, as
561 anaerobic fungal vegetative biomass (as opposed to the motile zoospores) is tightly attached
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563 to plant particles.
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566 Faeces samples are often used as reference samples to describe the microbial population of
567 the digestive tract, because the faecal microbiota might be expected to contain representatives
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569 from all regions of the large intestine. Our study provides evidence that horse faecal samples
570 can serve as qualitative reference samples, as almost all clades of anaerobic fungi found in
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572 the hindgut were also present in the faeces sample. The only exception was *Anaeromyces*,
573 which was only detected in some segments and at very low relative abundances. However,
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575 the relative abundance and concentrations of anaerobic fungi differed considerably along the
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577 horse hindgut and the functional implications of this variation requires further investigation.
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579 Differences in anaerobic fungal concentrations along the ruminant digestive tract is known to
580
581 occur (Davies et al., 1993). Furthermore, a cultivation based study by Griffith et al. (2009)
582 compared the anaerobic fungi in the rumen and faeces of the same cow, and found
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584 considerable differences in the abundance of the different taxa. Fungi with bulbous
585 morphotypes (*Caecomyces* and *Cyllamyces*) were the most abundant genera in fresh faeces,
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587 where they comprised a 5-fold greater proportion of the total fungal population than in the
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594 rumen. Polycentric morphotypes (*Orpinomyces* and *Anaeromyces*) were less frequently
595 isolated from fresh faeces as compared to rumen digesta. Conversely, a cultivation
596 independent study showed no difference in anaerobic fungal community composition in the
597 rumen, duodenum and faeces of three different cows (Jimenez et al. 2007).
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600 We acknowledge that no general conclusion can be made from the analysis of samples from
601 one horse, and the findings of this preliminary study should be used cautiously. Nevertheless,
602 the data presented here provide an important next step in revealing the considerable, as yet
603 largely uncharacterised, anaerobic fungal diversity in the equine hindgut. It also highlights
604 the need to conduct further studies to look at the activity, concentrations and diversity of the
605 anaerobic fungi throughout the digestive tract, due to evidence of differences between
606 hindgut segments.
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Figure captions

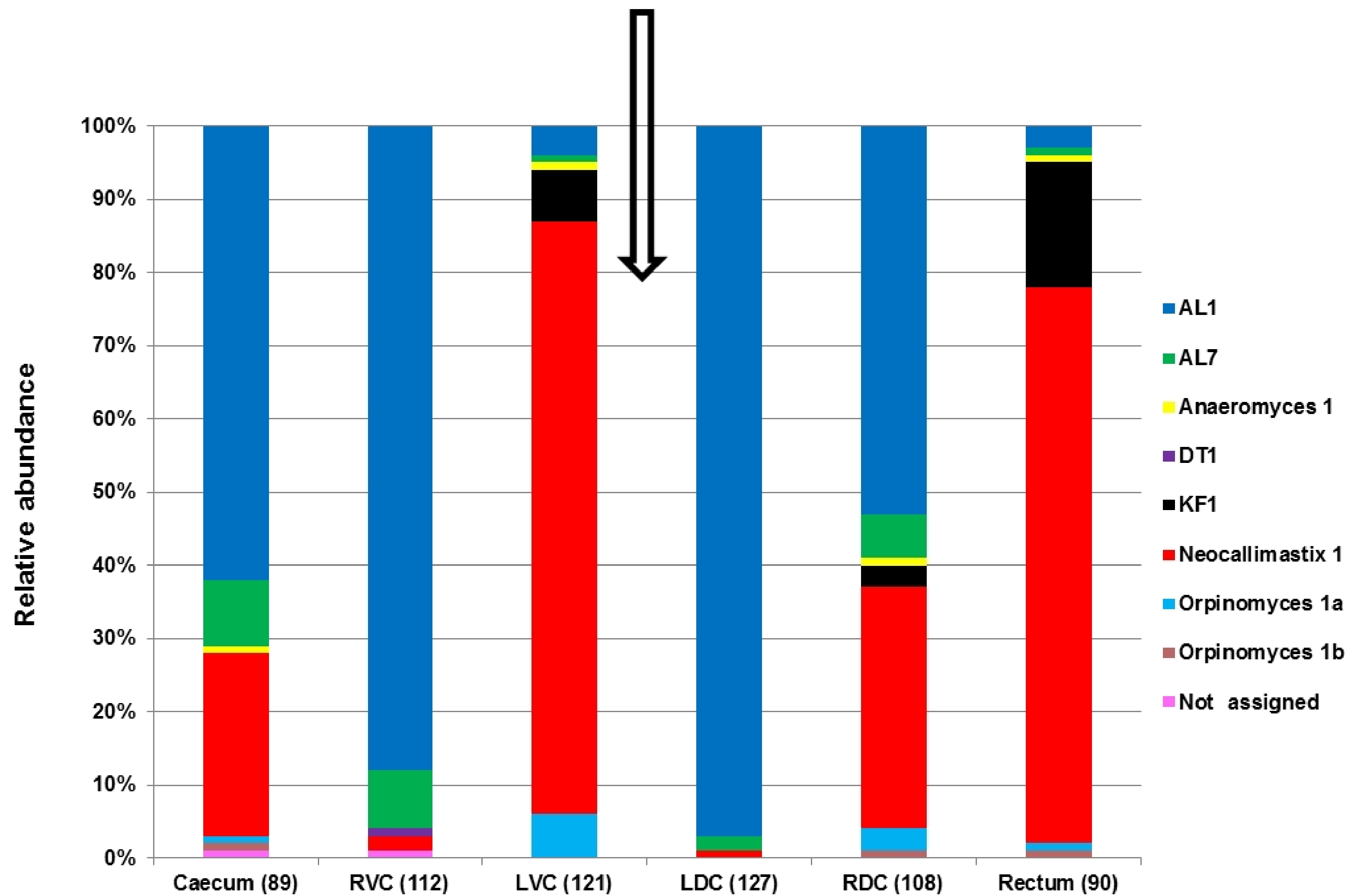
Fig. 1. Relative abundances of anaerobic fungal clades (as defined by Koetschan et al. 2014) in the ITS1 clone libraries constructed from six segments of the horse hindgut. The total number of clones in each library is indicated in parentheses on the x-axis. The arrow indicates the pelvic flexure position between the LVC and LDC.

Fig. 2. Quantitative profiles of anaerobic fungal clades (as defined by Koetschan et al. 2014) in the ITS1 clone libraries constructed from six segments of the horse hindgut. The top of the bars indicate mean concentrations (n=3) of total anaerobic fungal ITS1 gene copies in six different segments of horse hindgut, and different letters indicate significant differences ($P < 0.05$). Quantitative profiles were then generated from clade relative abundances and the

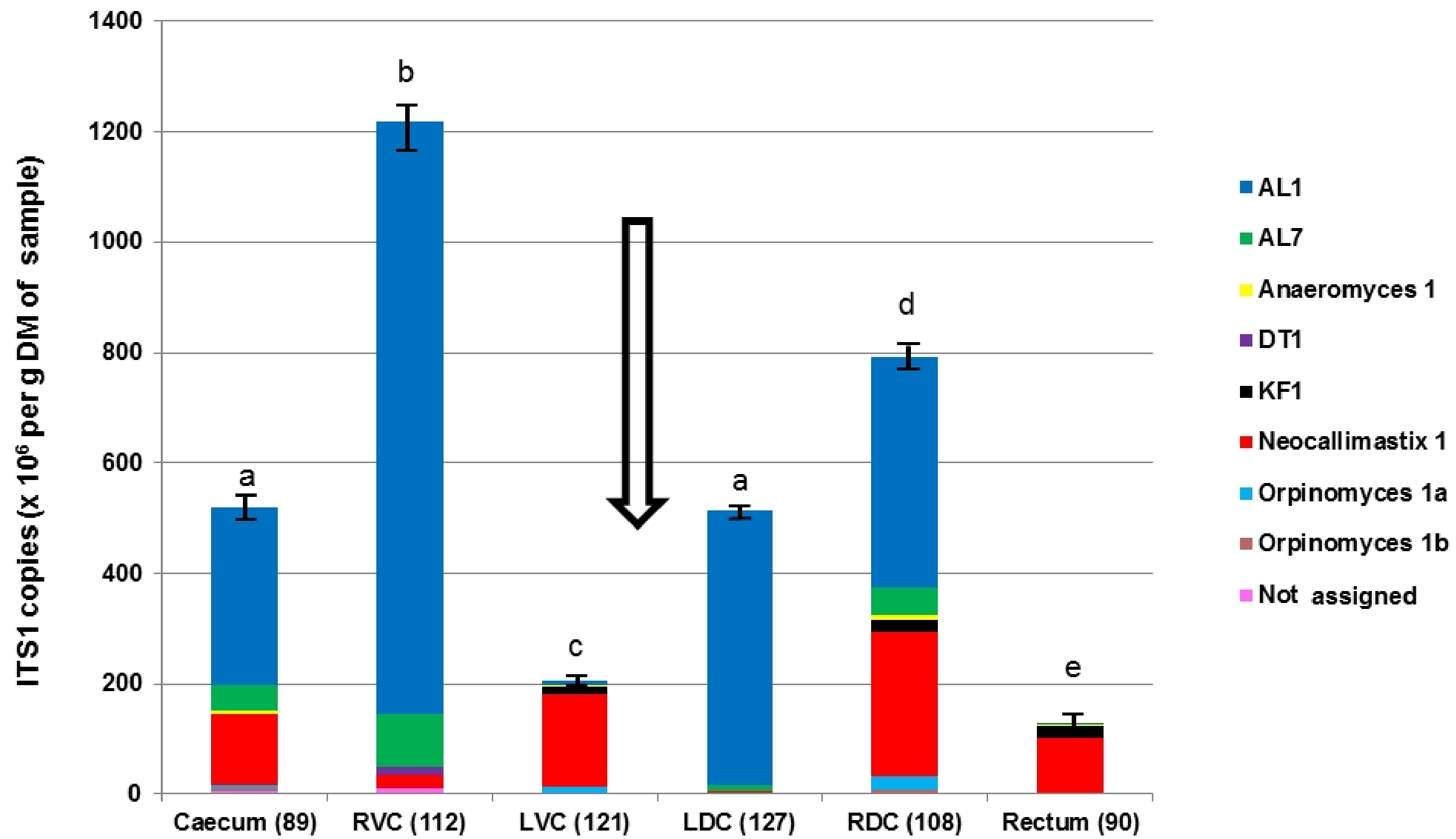
ITS1 concentrations in each segment. The arrow indicates the pelvic flexure position between the LVC and LDC.

Fig. 3. Phylogenetic relationships of ITS1 sequences of anaerobic fungi generated from the LDC inferred using the UPGMA method with bootstrap values from 1,000 replications. The evolutionary distances were computed using the Jukes-Cantor method. The analysis involved a total of 142 nucleotide sequences (127 sequences generated from the LDC in this study and 14 of the most closely related sequences plus 1 outgroup sequence).

Fig. 4. Phylogenetic relationships of anaerobic fungal LSU gene sequences generated from the LDC inferred using the UPGMA method with bootstrap values from 1,000 replications. The evolutionary distances were computed using the Jukes-Cantor method. The analysis involved a total of 45 nucleotide sequences (17 sequences generated from the LDC, 27 sequences of the cultured genera of anaerobic fungi plus 1 outgroup sequence).

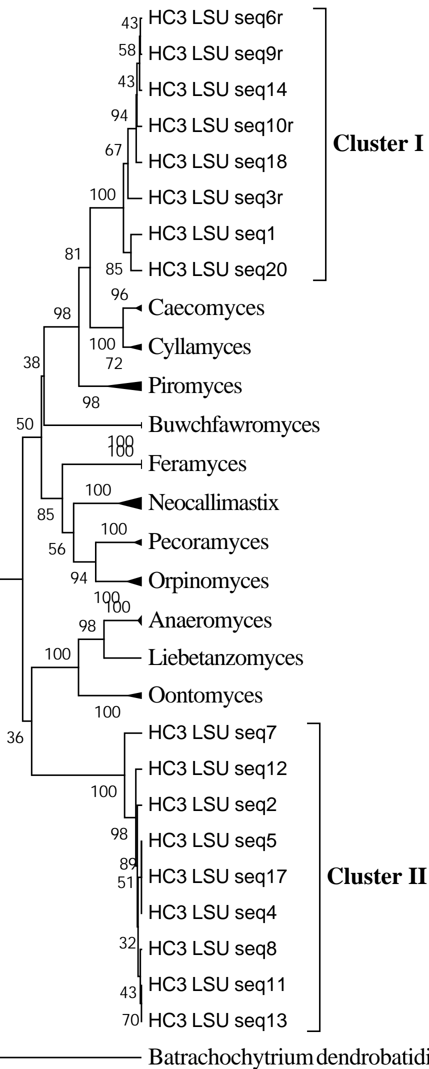


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Relative abundance
Caecum (89)
RVC (112)
LVC (121)
LDC (127)
RDC (108)
Rectum (90)
AL1
AL7
Anaeromyces 1
DT1
KF1
Neocallimastix 1
Orpinomyces 1a
Orpinomyces 1b
Not assigned



ITS1 copies (x 10^6 per g DM of sample)
1400
1200
1000
800
600
400
200
0
a
b
c
a
d
e
Caecum (89)
RVC (112)
LVC (121)
LDC (127)
RDC (108)
Rectum (90)
AL1
AL7
Anaeromyces 1
DT1
KF1
Neocallimastix 1
Orpinomyces 1a
Orpinomyces 1b
Not assigned

100
AL1
43
LDC ITS1 seq118
LDC ITS1 seq60
Uncultured Neocallimastigales clone WildAss01AOJA5 (GQ686305)
100
82
AL7
97
Caecomyces sp. W101 (DQ067604)
Caecomyces communis OF1 (KM878677)
100
LDC ITS1 seq175
Uncultured fungus clone 1F1-41 (JX184439)
Neocallimastix 1
60
64
Anaeromyces sp. JB-1999 isolate AUC2 (AF170188)
Anaeromyces sp. AMG-2014 isolate AIB16 (KF789509)
100
Issatchenkia sp. YS5 (AM233510)
0.3
0.2
0.1
0.0


HC3 LSU seq6r
HC3 LSU seq9r
HC3 LSU seq14
HC3 LSU seq10r
HC3 LSU seq18
HC3 LSU seq3r
HC3 LSU seq1
HC3 LSU seq20
Cluster I
Caecomyces
Cyllamyces
Piromyces
Buwchfawromyces
Feramyces
Neocallimastix
Pecoramyces
Orpinomyces
Anaeromyces
Liebetanzomyces
Oontomyces
HC3 LSU seq7
HC3 LSU seq12
HC3 LSU seq2
HC3 LSU seq5
HC3 LSU seq17
HC3 LSU seq4
HC3 LSU seq8
HC3 LSU seq11
HC3 LSU seq13
Cluster II
Batrachochytrium dendrobatidis (AY546693)
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58
43
94
67
100
81
85
96
98
100
72
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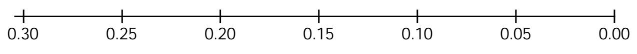
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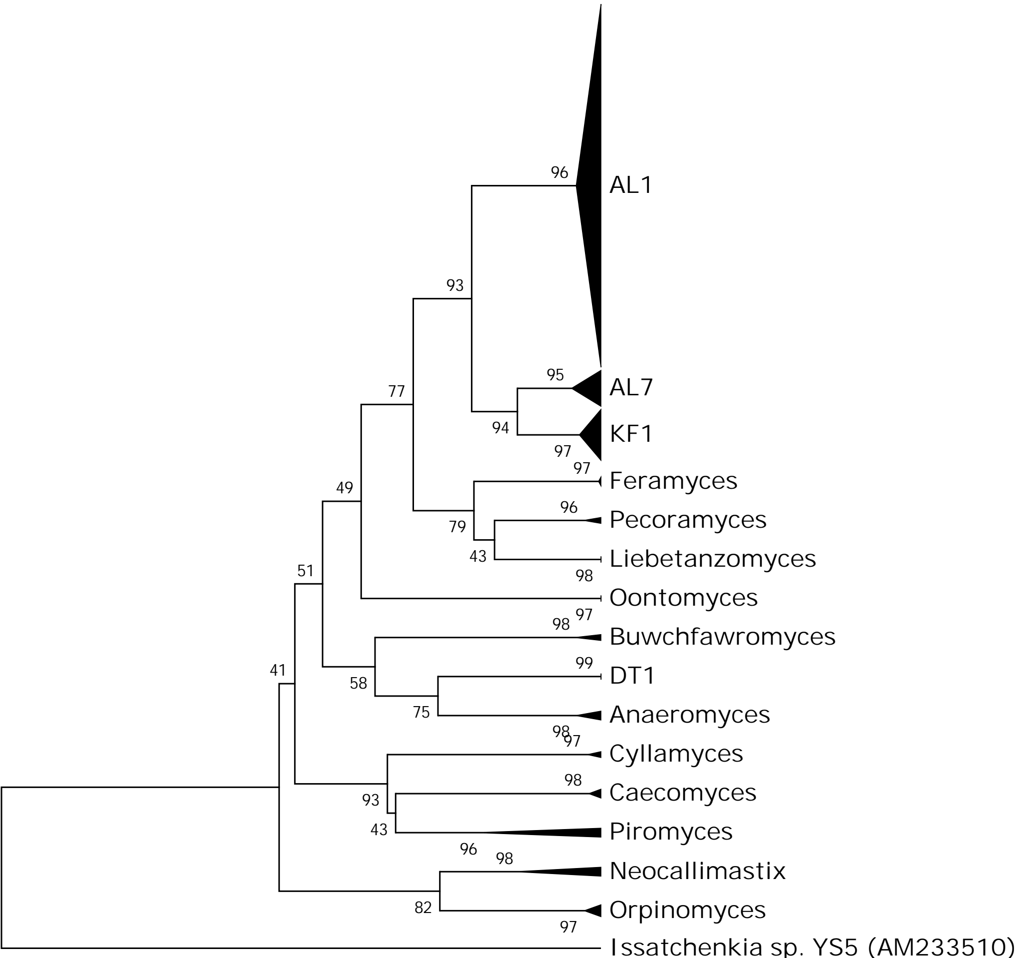
AL1
AL7
KF1
Feramyces
Pecoramyces
Liebetanzomyces
Oontomyces
Buwchfawromyces
DT1
Anaeromyces
Cyllamyces
Caecomyces
Piromyces
Neocallimastix
Orpinomyces
Issatchenkia sp. YS5 (AM233510)
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