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Equine anaerobic fungi: Key taxa of central importance to dietary fibre degradation

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Introduction

The hindgut microbiota of equines enables the degradation of dietary fibre, as the equine host lacks this enzymatic capability. The hindgut microbiota is comprised of five main groups of microbes: bacteria, anaerobic fungi, protozoa, archaea and viruses (Julliand & Grimm, 2016). Despite this, however, only bacteria tend to be routinely studied when analysis of the hindgut microbiota is undertaken. This is short-sighted, as anaerobic fungi play a unique role in fibre degradation. This is due to their combined invasive growth and potent enzymatic activity enabling them to disrupt plant structural barriers and access internal areas of the plant tissue that other microbes cannot (Orpin, 1975; Ho et al., 1988; Solomon et al., 2016). In addition to being the most effective fibre degraders in the herbivore gut (Lee et al., 2000), they also benefit other gut microbes by increasing the plant surface area available for them to colonise.

What are anaerobic fungi?

Shortly after anaerobic fungi were first isolated and described in ruminants (Orpin 1975, 1976, 1977a,b), they were also reported to occur in the hindgut of ponies (Orpin, 1981). However, their first published description in equines dates back much earlier, as they were first incorrectly classified as flagellated protozoa (Julliand & Grimm, 2016). Until the pioneering work of Orpin, it was universally accepted that all fungi contained mitochondria and respired aerobically. Therefore, anaerobic fungi were, and still are, unique in the fungal kingdom in that they are strict anaerobes that possess hydrogenosomes instead of mitochondria. Hydrogenosomes are highly specialized organelles that couple the metabolism of glucose to cellular energy production without the need for oxygen.

Anaerobic fungi are commonly found in the digestive tracts of mammalian herbivores and are the sole members of the class Neocallimastigomycetes within the phylum Chytridiomycota. All of the eleven characterised genera of anaerobic fungi (Table 1) represent just one family (Neocallimastigaceae) within this class. Cultivation independent techniques, however, have highlighted that numerous other anaerobic fungal taxa exist (Koetschan et al., 2014). Anaerobic fungi have also been detected in the non-mammalian herbivore gut and non-gut environments (Edwards et al., 2017). A recent phylogenetic census predicted that at least 34

anaerobic fungal genera and 274 species exist (Paul et al., 2018); with no doubt more to be discovered in the future as the range of environments they are detected in continues to expand.

Table 1: Morphological characteristics of the eleven currently characterised anaerobic fungal genera.

Genus	Thallus*	Zoospore#	Rhizomycelium	Reference for Type Species
Buwchfawromyces	MC	MF	Filamentous	Callaghan et al. (2015)
Oontomyces	MC	MF	Filamentous	Dagar et al. (2015)
Pecoramyces	MC	MF	Filamentous	Hanafy et al. (2017)
Piromyces	MC	MF	Filamentous	Orpin (1977a)
Liebetanzomyces	MC	MF	Filamentous	Joshi et al. (2018)
Feramyces	MC	PF	Filamentous	Hanafy et al. (In Press)
Neocallimastix	MC	PF	Filamentous	Orpin (1975)
Anaeromyces	PC	MF	Filamentous	Breton et al. (1990)
Orpinomyces	PC	PF	Filamentous	Barr et al. (1989)
Caecomyces	MC	MF	Bulbous	Gold et al. (1988)
Cyllamyces	PC	MF	Bulbous	Ozkose et al. (2001)

* Monocentric (MC) or polycentric (PC) thallus
# Monoflagellated (MF) or polyflagellated (PF) zoospores

How do anaerobic fungi grow?

To understand why anaerobic fungi have a unique capability to degrade fibre, we need to understand their life cycle (Figure 1). The anaerobic fungal life cycle comprises both motile and vegetative stages (see review of Gruninger et al., 2014). Motile zoospores are released from mature sporangia upon the detection of new substrates, which are then subsequently located via chemotactic signals. The zoospores then encyst and germinate on the substrate and form an extensive rhizodial system and sporangium. This simple scheme of events that characterizes the life cycle of monocentric fungi becomes more complex for polycentric fungi that develop multiple thalli. Furthermore, a third stage of the anaerobic fungal cycle has been proposed that is characterised by the formation of oxygen- and desiccation-tolerant structures that are stable for long periods of time. These structures are believed to be important in the transfer of anaerobic fungi between animals. This third stage, however, has not been well characterised to date, and it is likely that the mechanisms and/or type of structures are not consistent across all anaerobic fungi (Figure 1).

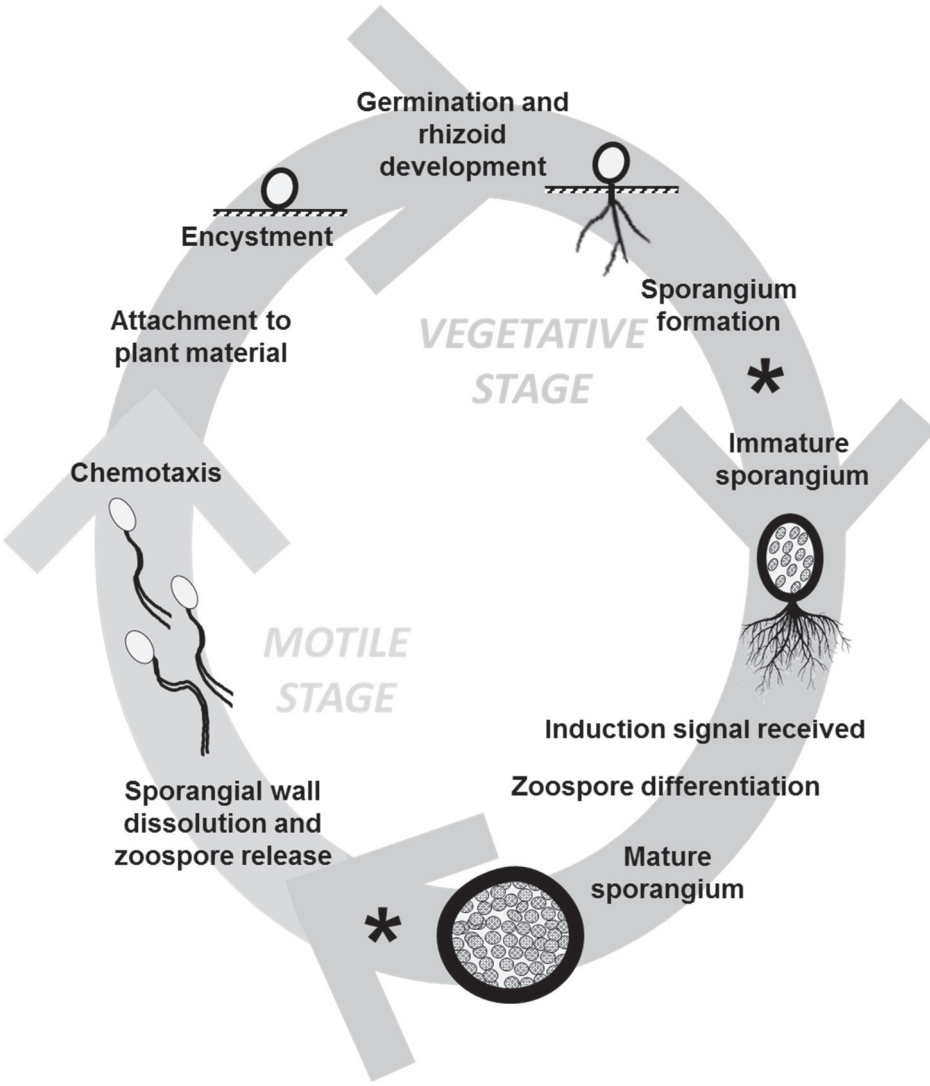

Figure 1. A schematic of the life cycle of a monocentric fungus modified from Gruninger et al. (2014). The stages where 'resistant' structures tolerant to oxygen and desiccation (that have been reported to date) may be formed are indicated (*).

In the rumen, the highest zoospore density is reached within 30–60 min of feed ingestion (Orpin, 1975, 1976, 1977a), with zoospore release triggered by haem and other related porphyrins from the freshly ingested plant material (Orpin & Greenwood, 1986). However, the cause of zoospore release in the equine hindgut is unclear, as it is not known if these triggering compounds can survive passage through the equine stomach and small intestine. Furthermore, released zoospores chemotactically locate freshly ingested plant material using soluble sugars (Orpin & Bountiff, 1978) and/or phenolic acids (Wubah & Kim, 1996). Again, it is unclear to what extent these same chemotactic signals are available and/or used by anaerobic fungi in the equine hindgut.

Why are anaerobic fungi important?

Zoospore numbers in the equine hindgut are low (3.2-4.7 x 10^4 zoospores/ml caecal contents; Orpin, 1981) compared to bacteria, however, this measure does not reflect their biomass. Similar zoospore numbers in the rumen have been shown to correlate with anaerobic fungi representing up to 20 % of the total microbial biomass (Rezaeian et al., 2004). Therefore, their biomass in the equine hindgut is significant.
The quantitative contribution of anaerobic fungi to fermentation in the equine hindgut is not currently known, however, it has been shown in ruminant studies that anaerobic fungi increase dry matter digestibility by 7-9% (Gordon & Phillips, 1998). Beneficial effects on fibrous feed intake are also known, with the extent of the benefit primarily related to the quality of the forage. For example, removal of anaerobic fungi caused a 40% decrease in feed intake in one study where forage with a high lignocellulose content was fed (Gordon & Phillips, 1993). The increase in feed intake is thought to be due to the anaerobic fungi resulting in a more rapid clearance of digesta from the rumen, as a consequence of their physical disruption of fibrous particles (Gordon & Phillips, 1998). If also true in equines, these effects are clearly of benefit, particularly when replacing energy dense concentrates for more bulky fibrous feeds or with animals that have poor dentition.

Which anaerobic fungi are present in equines?

Since their initial description, different anaerobic fungal genera and species have been reported in the equine hindgut including *Piromyces equi* (Orpin 1981; Freelove et al., 2001), *Caecomyces equi* (Gold et al., 1988), *Piromyces citronii* (Gaillard-Martinie et al., 1995), *Piromyces mae* (Li et al., 1990), *Bwachfawromyces eastonii* (Callaghan et al., 2015) and *Piromyces finnis* (Solomon et al., 2016). Early cultivation studies primarily classified anaerobic fungal genera in terms of their morphology (Figure 2), for example thallus morphology, flagellation of zoospores and rhizoidal structure. However, this has now been shown to be inadequate for differentiating anaerobic fungi even at the genus level (Table 1). Furthermore, some cultures display pleomorphism in terms of their sporangial and rhizoidal structures (Joshi et al., 2018). Therefore, many of the previously characterised equine isolates (many of which unfortunately are no longer available in culture) may actually be found to represent different genera if their identity was reassessed using a combination of both genetic and morphology based approaches.
Cultivation independent analysis has furthermore recently indicated that the anaerobic fungi in equines may be distinct from that of other mammalian herbivores. In a survey of domesticated and captive wild herbivores, Liggenstoffer et al. (2010) showed that most of the equines sampled (including horses, zebra and a Somali wild ass) were dominated by two novel uncultivated genera, namely NG1 and NG3. However, it appeared that these two taxa were not exclusive to equines as they were also found in other animals (Liggenstoffer et al., 2010). These uncultivated genera were subsequently renamed to AL1 and AL3 (Koetschan et al., 2014).

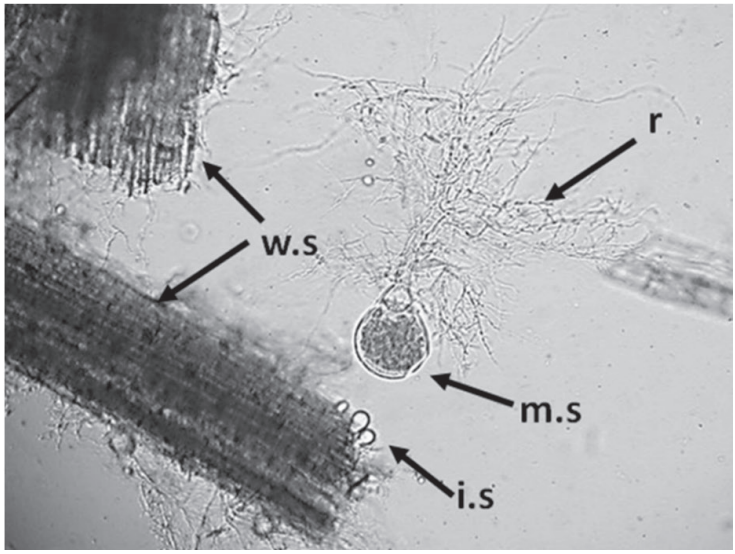

Figure 2. A light microscopy image of a monocentric anaerobic fungus with a filamentous rhizomycelium (r) cultured on wheat straw (w.s.). A mature sporganium (m.s.) can be seen that has developed using soluble nutrients in the medium, as well as immature sporangia (i.s.) developing on the particle of wheat straw. Image kindly provided by Dr Tony M. Callaghan (Aberystwyth University, Wales, UK).

How do equines acquire anaerobic fungi?

As foals consume faeces, a behaviour called coprophagy, it is likely that they acquire anaerobic fungi through this route. This is because ruminant studies have shown anaerobic fungi can survive in air-dried faeces for long periods of time due to the previously mentioned oxygen- and desiccant-tolerant structures (Davies et al., 1993; McGranaghan et al., 1999). Consistent with this suggestion is the observation of structural bodies, similar to resting cysts, in cultures of an equine anaerobic fungal isolate (Orpin, 1981). Anaerobic fungi have also been shown to survive in saliva (Lowe et al., 1987), although this mode of transfer is likely to be of greater importance in ruminants compared to equines due to rumination.

Microscopic evidence has shown that anaerobic fungi can be detected in foal faeces within the first few weeks of life (Julliand et al., 1996). More recently, a molecular based study established that anaerobic fungi could not be detected in foal faeces until 14 days after birth, one week later than the detection of protozoa (Hubball et al., 2014).

What influences anaerobic fungi in the equine hindgut?

In the rumen, it is known that anaerobic fungi are affected by a variety of factors including diet and interactions with certain microbes (Gordon and Phillips, 1998). It is likely that a lot of this rumen-based information, however, cannot be directly translated to anaerobic fungi within the equine hindgut. This due to the fundamental difference between ruminants (where freshly ingested feed enters the rumen) and equines (where feed first passes through the stomach and small intestine before reaching the hindgut) in terms of the main gut site where fibre degradation primarily occurs. As such, this section will focus only on studies that have looked at the effects of different factors on anaerobic fungi either directly in the equine hindgut or using equine anaerobic fungal isolates.

A preliminary study showed that the animal variation in equine anaerobic fungal concentrations is sizable (Birch et al, 2011). In two different sampling periods (P1 and P2), one horse had consistently a larger concentration of anaerobic fungal DNA per g dried faeces (212 and 64.0 ng for P1 and P2 respectively) compared to the other three horses (< 3.96 ng) that grazed the same pasture (Birch, 2011). Interestingly, all four horses had the same anaerobic fungal population detected in their faeces (Birch, 2011). As the study of Dougal et al (2012) found that concentrations of anaerobic fungal DNA in the caecum, right dorsal colon and faeces did not significantly differ, it is likely that anaerobic fungal concentrations within the caecum and colon will also be influenced by animal variation. However, it has also been reported that anaerobic fungal concentrations were >10 fold higher in the colon compared to the caecum (Moore & Dehority, 1993). In a more recent study, large differences in anaerobic fungal concentrations were also found to occur along the equine hindgut (Mura et al., In Press). The reason for the contrasting reports regarding the effect of gut site on anaerobic fungal concentrations is not clear, and it is speculated that dietary differences between the studies may be responsible.

Knowledge on the effect of diet on anaerobic fungal concentrations in the equine hindgut is very limited. However, one study reported that decreasing the amount of concentrate from 40% of the diet to 10% did not significantly change the caecal or colonic concentrations of anaerobic fungi (Moore & Dehority, 1993). However, mean values nearly doubled in the low concentrate diet compared to the high concentrate diet (Moore & Dehority, 1993). In ruminants, the inclusion of concentrates in the diet can result in different effects, and it has been speculated that this is because not all anaerobic fungi are able to degrade starch (Gordon & Phillips, 1998). *Piromyces citronii,* which was isolated from the equine hindgut, has been shown to be able to utilise starch (Gaillard-Martinie et al., 1995), as well as two out of three other equine isolates in another study (Orpin, 1981). This ability, however, has not been assessed in the other equine isolates characterised to date (Gold et al., 1988; Li et al., 1990; Callaghan et al., 2015, Solomon et al., 2016).

Whilst it is clear that not all anaerobic fungi can utilise starch, this is not the case for simple sugars such as glucose and cellobiose which are universally utilised by anaerobic fungi. However, it is important to note that the presence of glucose has been shown to repress gene expression of plant biomass-degrading enzymes in the equine isolate *Piromyces finnis*, as well as other anaerobic fungi (Solomon et al., 2016). Therefore, the presence of glucose in the equine hindgut will likely limit the ability of anaerobic fungi to degrade plant material, although this catabolic repression is reversible (Solomon et al., 2016).

Despite glucose being universally utilised, differences exist between anaerobic fungi in terms of their growth on this substrate, as well as other more complex substrates (Hanafy et al., In Press). For example growth of *P. finnis* on plant biomass was greater than that of two other anaerobic fungi isolates for certain C3 and C4 grasses (Solomon et al., 2016). Another study showed that, relative to comparable ruminal isolates, equine *Piromyces* isolates utilised glucose more rapidly and degraded cellulose faster and to a greater extent (Julliand et al., 1998). These superior growth and metabolic characteristics are likely to enable equine anaerobic fungi to cope better with the shorter residence time and more limited nutrients available in the equine hindgut compared to the rumen. Indeed, these characteristics may offer equine anaerobic fungi an ecological advantage in colonising

other mammalian herbivores. This may partly explain why the AL1 and AL3 anaerobic fungal clades reported to be dominant in the equine hindgut in the Liggenstoffer et al (2010) study were also detected in other types of herbivores. Interestingly, however, it has recently been shown in a preliminary study that the anaerobic fungal community composition along the hindgut is not consistent (Mura et al., In Press). The reasons for this is not clear, although the finding is not surprising considering differences in the community composition along the hindgut has been previously reported for bacteria, protozoa and archaea (Julliand & Grim, 2016; Fliegerova et al., 2016).

Conclusion and take home message

Anaerobic fungi play a unique and important role in dietary fibre degradation, and are a significant part of the normal hindgut microbiota of equines. Whilst much of the knowledge base generated from ruminants can be translated to equine anaerobic fungi, it is clear that further research is needed to quantify their contribution to fibre degradation in the equine hindgut as well as how their activity is influenced by dietary factors.

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