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DIVERSITY OF ANAEROBIC FUNGI POPULATION (PHYLUM NEOCALLIMASTIGOMYCOTA) IN THE HORSE HINDGUT

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Alla mia Famiglia e alla mia nonnina

La scienza intimorisce solo chi non la conosce. Ma in realtà la scienza è l'unica cosa che distingue l'homo sapiens dal resto delle creature viventi. Va coltivata, non certo bloccata.

- Rita Levi Montalcini -

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ABSTRACT

This thesis aims to study the diversity of symbiotic gut anaerobic fungi (phylum Neocallimastigomycota) in the different segments of the equine hindgut (cecum, right ventral colon, left ventral colon, left dorsal colon, right dorsal colon, and rectum). A clone library of fungal internal transcribed spacer region-1 (ITS1) fragments of rDNA was prepared for each hindgut segment. The ITS1 fragments were sequenced and phylogenetically analysed.

The major finding of this study is the prevalence of uncultured Neocallimastigales in the horse lower digestive tract, representing 81% of the hindgut fungi and the completely different diversity of gut fungi of feces compared to cecum and colon content. The fecal sample did not reflect the factual microbial population composition of hindgut. The fungal population similarity between LVC and feces was evidenced in this study. RVC, LDC, and RDC are inhabited by unknown gut fungi. A significant proportion of uncultured sequences is highly similar to the sequences of a new group of anaerobic fungi, which is specific for the Equidae family. Moreover a new cluster of anaerobic fungi was found in the digestive tract of horse (Anglo-Arabian reared in Sardinia). The horse digestive tract is populated by unknown, not yet cultivated species of anaerobic fungi and only a limited percentage of identified sequences can be classified into known species of gut fungi. The conventional microbiological cultivation techniques underestimate the diversity of the anaerobic fungal population in the horse hindgut.

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Introduction

Microorganisms represent the largest component of biodiversity in the world. Coevolved symbiotic relationships between microorganisms and multicellular organisms are a prominent feature of life on Earth. All animals, including humans, are adapted to life in a microbial world. Anaerobic habitats have existed continuously throughout the history and the gastrointestinal tract always represented a microbial microniche. A very large collection of microorganisms thus resides in the intestine. Microorganisms colonize and grow rapidly under the favorable conditions of the gut and therefore they can compete for nutrients with the host (Mackie, 2002). This microbial challenge has modified the course of animals evolution, resulting in the selection of complex animal-microbe relationships, which vary according to type of digestive tract. Foregut fermentors comprise animals with a pregastric fermentation chambers as the rumen, reticulum, and omasum of ruminants and diverticula or fermentative sacs of other ruminant-like mammals. Hindgut fermentors are defined as those animals with large fermentation compartments in the cecum, colon and rectum. Large populations of microorganisms inhabiting the gastrointestinal tract are represented by all major groups of microbes including bacteria, archaea, ciliate protozoa, anaerobic fungi and bacteriophages. This microbial community is characterized by high population density, wide diversity and complexity of interactions and is now considered as metabolically adaptable and rapidly renewable organ of the body, which plays a vital role in the normal nutritional, physiological, immunological and protective functions of the host animal (Mackie 2002).

The microbiota of the adult large mammals is found primarily in the distal intestine and consist of $>10^3$ microorganisms, comprising nearly 500 species. At the birth, the intestine of mammals is sterile, but it is rapidly colonized by microorganisms from maternal and environmental sources (Neu et al., 2007). The number and species of microorganism in the different gut segments are affected mainly by the pH and the retention time of digesta. The low pH of gastric content and the rapid transit of digesta

through the midgut or small intestine tend to inhibit the growth of many microbes. However, the relatively neutral pH and prolonged retention of digesta in the foregut of some animals and hindgut or large intestine of all terrestrial vertebrates are accompanied by an increase in the number and variety of bacteria and, in some animal species, the additional presence of protozoa and fungi. The bacteria concentration, in this part, is similar to those in the rumen of polygastric animals (Stevens and Hume 2008).

The 80% of mammals are herbivores. To access the complex carbohydrates present in plants, they usually have enlarged and/or elongated digestive tracts, often including fermentation chambers or sacs in the foregut or hindgut. Cecum-colon (hindgut) fermenters represent an older differentiation than foregut fermenters, which in turn are older than ruminants (Langer, 1991). Bacteria, protozoa and anaerobic fungi inhabit these enlarged gut compartments as well as other sites of the gastrointestinal tract. The fermentative activity of these microbes results in the production of volatile fatty acids, often termed as short-chain fatty acids (SCFA), most notably, acetate, propionate and butyrate, but even isobutyric, isovaleric and valericacid which are absorbed by the host animal and make a variable and in some cases considerable contribution to its nutritional economy as energetic source.

The best studied herbivores are ruminants, while microbiota of gastrointestinal tract of hindgut herbivores is less studied, which is also the case of equine cecum-colon ecosystem. Despite the importance of the intestinal microbial ecosystem for host animal health and performance, information regarding the microbial ecology of the equine digestive tract is very limited. Recent research indicated that the anatomical segments of the equine hindgut exhibit differences in microbial population and fermentative activity, namely between the cecum and the colon of horses (Moore and Dehority, 1993; Julliand et al., 2001). Moreover, the implication of the right ventral colon ecosystem in forage degradation has been highlighted (de Fombelle et al., 2003). To our knowledge there are however no studies on the diversity of anaerobic fungi in the different anatomo-physiological segments of the horse hindgut and the significance and the contribution of gut fungi to the fermentative activity have not yet been fully understood, and basically

are unknown. The target of this study is therefore concentrated on the anaerobic fungi of class Neocallimastigomycetes of equine hindgut with the aim to compare diversity of gut fungi in different segments of caeco-colic system and evaluate the possible fermentative role of fungal population. The better understanding of the microbial diversity of the equine hindgut is essential for improving our knowledge of digestive processes, for the implementation of proper feeding practices and the future prevention, animals welfare and treatment of diseases involving the gastrointestinal tract as laminitis or grass sickness.

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CHAPTER 1

THE HORSE: Anatomy of the digestion system and feeding

Introduction

Horse is odd-toed ungulate of the order Perissodactyla, family Equidae, genus Equus. Distinctive characteristic of the order Perissodactyla (comprising horses, tapirs, and rhinoceros) is the development of the teeth, the lower limb with the peculiar plan of the carpus and tarsus bones and the evolution development of the hindgut into chambers for fermentation of ingesta (Frape, 2004). Equidae (sometimes known as the horse family) is the taxonomic family of horses and related animals, including the extant horses, donkeys, and zebras, and many other species known only from fossils. Horse is free-ranging herbivore of grassland environments adapted to eat large quantities of high fiber diet to obtain energy and nutrients necessary for its survival. Life on open pasture for millions of years is in contrary with present practices of horse breeding. Nowadays, with the increase of their use in sport, many horses are housed during long period of time and fed daily with two or three meals of forages plus concentrate. This feeding management is not as expected by the horse's phylogenetic adaptation (Janis, 1976). The management procedures have important implications on the utilization of nutrients from both concentrate and forage components of the total ration, on the digestive tract in general and the hindgut in particular and, of course, on the health and welfare of the horse (Hill, 2007). Gastrointestinal disease is a leading cause of morbidity and mortality of horses, and a wide range of clinical conditions alter the gastrointestinal microbial population (microbiota), including various types of colic, colitis, and laminitis (Costa and J. Scott Weese, 2012). The absence of rumen in the gastrointestinal tract of horse is compensated by voluminous cecum and colon, where the microbial fermentation of plant biomass occurred (Stevens and Hume, 1998).

1.1 Anatomy of the digestive system

The horse's digestive system (Fig.1) is considered to intermediate the non ruminant and ruminant type of fermentation in terms of high rates of enzymatic digestion occuring in the foregut (mouth to ileum) plus high rates of fermentative microbial digestion occuring in the hindgut (cecum to rectum) (Jones FSA3038). Inadequate knowledge of the horse gastro-intestinal tract (GI) and basic digestive physiology is the root of many feeding errors involving both nutritional management and feed formulation.

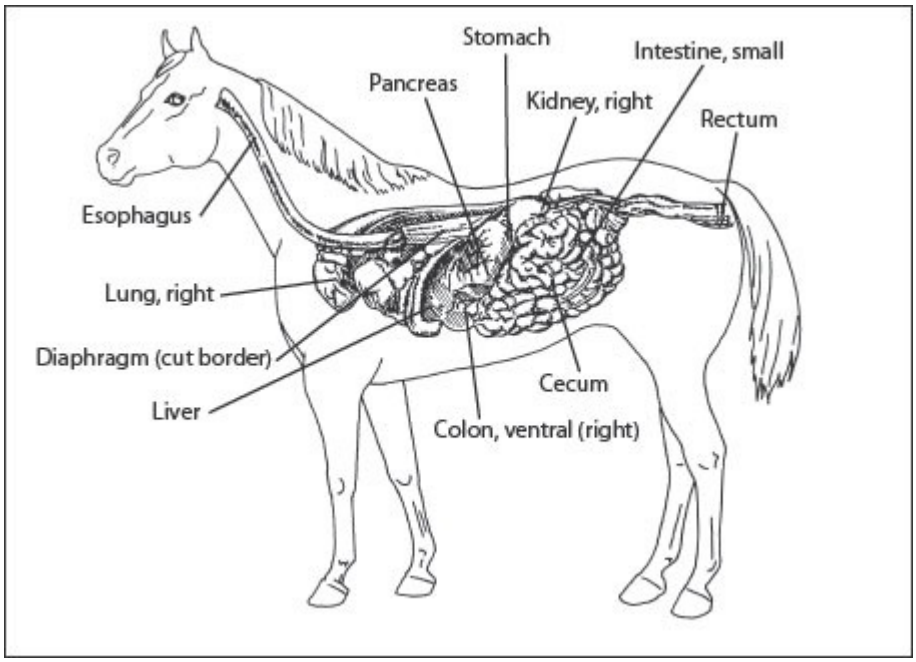

Figure 1 - Horse's digestive system

The foregut includes the mouth, the esophagus, the stomach and small intestine, whereas the hindgut includes the cecum, the large colon, the small colon and the rectum. So the digestion of food and passage of digesta require the secretion of large quantity of fluid and electrolytes by the salivary glands, stomach, pancreas, biliary system and intestine. The continuous feeding and greater microbial fermentation capacity require larger volumes of fluid, much bigger than in carnivores and omnivores (Stevens and Hume, 1998).

1.1.1 The mouth

The digestive process begins with the prehension of food. The lips, tongue and teeth of the horse are ideally suited for the prehension, ingestion and alteration of the physical form of feed to that suitable for propulsion through the gastrointestinal tract in a state that facilitates admixture with the digestive juices. Feed is supplied as fresh pasture, long hay or variations of stored forage and as concentrate feed containing processed or unprocessed cereal grains. The upper lip is strong, mobile and sensitive and is used during grazing to place forage between the teeth. More intensive mastication by the horse means that the ingestion rate of long hay, is three or four times faster in cattle and sheep than it is in horse or ponies, although for long hays the number of chews per minute, according to published observations, is similar (73-92 for horse and 73-115 for sheep) (Frape, 2004). The mature horse has 18 upper and 18 lower teeth consisting of six upper and six incisors and 12 upper and lower molar (Jackson S.G.). The teeth, reducing hay and grass particles to less than 1.6 mm in length, participate in the digestive process primarily and provide a greater surface area for the action of digestive juices. The number of chewing movement for roughage is considerably greater than that required for chewing concentrates. Horse make between 800 and 1200 chewing movements per 1 Kg of concentrates, whereas 1 Kg of long hay requires between 3000 and 3500 movements (Frape, 2004). Some study demonstrated that, different dental architecture between horse and sheep, induces a different degree of degradation of food and, as a consequence, a different mean particle size in feces (Hill, 2007). The chewing also stimulates the flow of saliva which may initiate chemical digestion of feed and lubricates feed prior to its passage to the remainder of the tract. The evidence has shown that abnormal or diseased teeth can cause digestive disturbances and colic. The apparent fibre digestibility, the proportion of fecal short fibre particles and plasma free fatty acids were all increased after dental correction of mars (Ralston et al., 2001).

1.1.2 Saliva

The presence of feeding material stimulates the secretion of a copious amount of saliva. This fluid seems to have no digestive enzyme activity, but its mucus content works as an efficient lubricant preventing "choke". The concentration of bicarbonate and sodium chloride in the saliva is directly proportional to the rate of secretion and is increased during feeding. The continuous secretion of saliva during eating seems to buffer the digesta in the proximal region of the stomach (Frape, 2004). Horses fed with two different diets (high fiber pellet and high cereal pellet) produce a different volume of saliva and, consequently, a different pH in the stomach (de Fombelle et al., 2003).

1.1.3 The stomach

Horse stomach is relatively small organ (compared to the capacity of the entire tract) and cannot accommodate large quantities of food at one time (Fig. 2). It contains different regions with different functions and physiological conditions, with the pH ranging from 2.5 to 5.6 with a high fibre low protein diet (Argenzio et al., 1974). Limited enzymatic digestion alimentary proteins and peptides by the pepsin, and some fermentative digestion performed by small microbial population occurs in the stomach. Food remains in the stomach only about 15 minute before it starts to pass into the small intestine (Jones FSA3038), but it is rarely completely empty and a significant proportion of digesta remains in stomach for 2 - 6 hours. When fresh digesta are offered, the stomach initiates soft peristaltic contractions and most of the old digesta pass into the duodenum (Van Weyenberg, 2006). When digesta approaches the pylorus, the pH falls, due to the HCl secretion, potentiating the proteolytic activity of pepsin and stopping the microbial fermentation. In particular, the pH is significantly more acid with the high fibre pellet (de Fombelle et al., 2003). Moreover there is a significant amount of lactic acid produced from the fermentation of soluble sugars by microbes located in the region of the stomach (Varloud et al., 2007). The degree of protein digestion is minimal, due to the small size of the stomach and the relatively short retention time (Jackson S.G.).

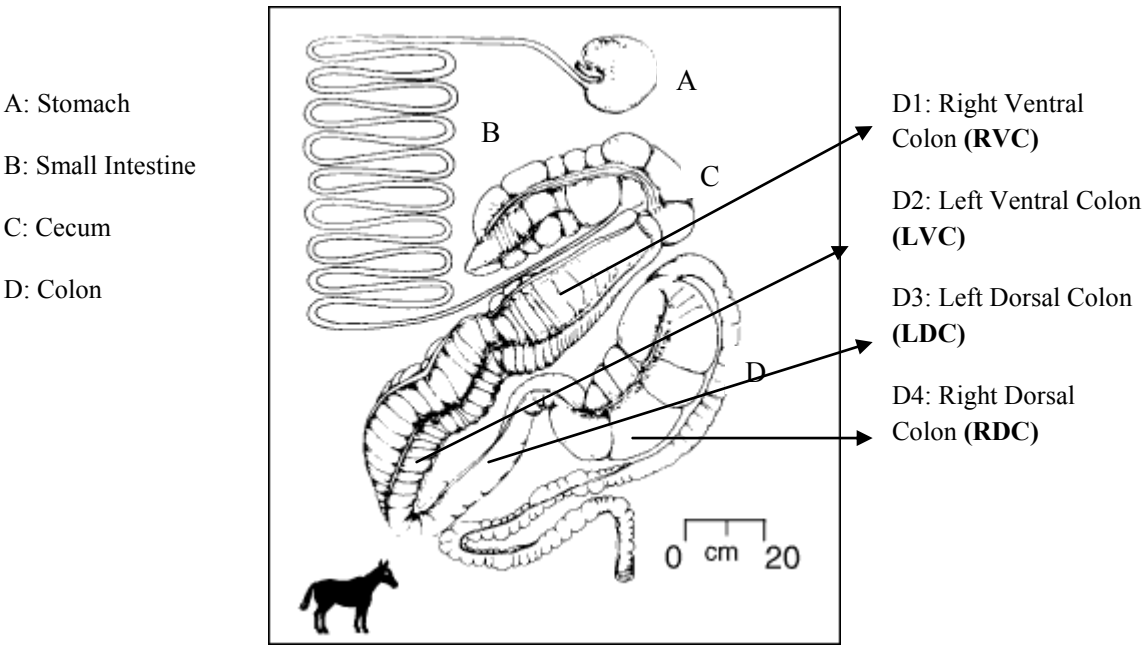

Figure 2 - Horse's digestive tract

According to the de Fombelle et al. (2003), in the stomach, irrespective of diet, there is a highest concentration of lactobacilli, streptococci and lactate utilizing bacteria. This finding suggests an important participation of these microorganisms in the degradation of easily fermentable carbohydrates.

1.1.4 Small Intestine

The small intestine is composed of duodenum, jejunum and ileum, were the pH is 6.3, 7.1, 7.5, respectively (Markie and Wilkins, 1988). It is 21 meters long organ and has a capacity of about 56 litres of ingesta. It receives a continuous flow of pancreatic juice and represents the major site for the digestion and adsorption of sugars, proteins and fats (Jackson, S.G.). Rosenfeld and Austbø (2009a) showed that feed-technological treatment of cereals can improve digestibility of starch and protein in the precaecal compartments of the equine GI-tract and thus increases their nutritional value and minimizes the risk of colic and laminitis. The walls of the gut contain longitudinal and circular muscle fibres essential for the contractions necessary for moving of the digesta by the peristaltic

process into the ultimate part of the anus. The peristaltic contraction is also important for the thorough admixture of digestive juices and for bathing the absorptive surfaces of the wall with the products of digestion (Frape, 2004). Large quantities of pancreatic juice are secreted as a result of the presence of food in the stomach in response to stimuli mediated by vagal nerve fibres, and by gastric HCl in the duodenum stimulating the release of the polypeptide hormone secretin into the blood. Although secretion is continuous, the rate of pancreatic juice secretion increases by four to five times when feed is first given. Pancreatic juice entering the duodenum has low enzymatic activity, but provides large quantities of fluid and sodium, potassium, chloride and bicarbonate ions. Some active trypsin is, however, present. The buffering of digesta is important due of large microbial intestinal production of volatile fatty acids (VFA). The secretion of pancreatic juice and bile is complete in 48 hours. Bile helps to preserve an optimal condition in the intestine for the activity of digestive enzymes (Frape, 2004). The digesta passage rate is very rapid in the small intestine, with most of the digesta rate being near 30cm/min (Van Weyenberg, 2006). The lower frequency of the contractions is localized in the segment farther from the pylorus (14–15 movements/min in the duodenum versus 10–11 movements/min in the ileum). Distension of the stomach increases the frequency of ejection and stimulates the passage of digesta from the small intestine into the cecum, because of increased motility of the ileum. This phenomenon is called the gastroileal reflex and is under neural control (Van Weyenberg, 2006). The rate of digesta passage through the small intestine varies with feed type and different physiological conditions. In small intestine the 100% of cytoplasmic carbohydrates are digested into glucose and lactate (85% of starch and 5% - 15% of cell wall carbohydrates) (Santos et al., 2011). It is important to highlight that the efficiency of carbohydrate digestion in the small intestine is important the increase of the energy available to the horse and the decrease of the potential of colic caused by excessive carbohydrates reaching the hindgut (Jones FSA3038). 90% to 95% of lipids are digested and the amount of long-chain fatty acids absorbed from the small intestine is 90-95% of digested lipids (Santos et al., 2011). About 50 to 70% of the protein in grain-based diets is digested to amino acids and adsorbed from the small intestine, but less than one-third of hay protein is adsorbed from

upper tract. The fat soluble vitamins A, D, E and K are also adsorbed in this tract along with B vitamin, calcium and phosphorus (Jones FSA3038).

1.1.5 Hindgut

Large intestine of horses enables longer retention of feed and microbes, which could in turn promote fibre digestion. It includes the cecum, large colon, small colon and rectum. The material not digested in the foregut passes through the ileo-cecal orifice into the cecum (Fig. 3). Cecum and colon are analogous to the rumen and reticulum of cow and sheep, and house billions of bacteria, protozoa and fungi which are in symbiotic relationship responsible for the digestion of cellulose and other fibrous fractions of the feed (Jackson S.G.). This microbial community produces volatile fatty acids (VFA), which represent a source of energy for the host animal (Kobayashi et al., 2006). The microbial population and activity within different parts of the gut is dependent both on the anatomy of the gut and the supplied substrate, which reachs that part of the gut. Under normal feeding conditions, the microbial population maintains a balance with the host, keeping the integrity of the ecosystem, contributing to the prevention of disorders and forming a barrier against pathogens (Santos et al., 2011).

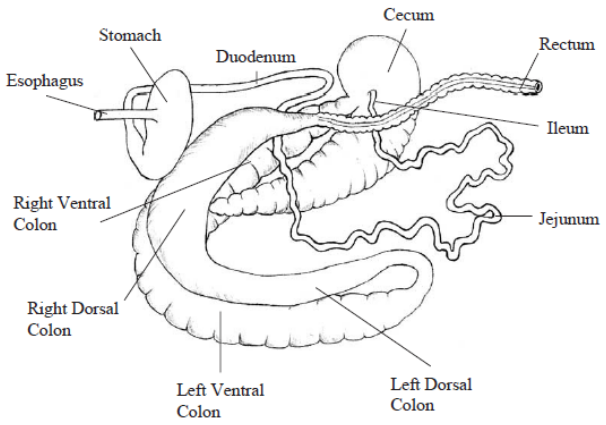

Figure 3 - Localization of main selective retention sites in the horse hindgut

1.1.5.1 Cecum

The first part of the large intestine, where the undigested material runs into, is the cecum. It has a volume of 52-60 L (Varloud et al., 2004). The digesta reaches it within 2-3 hours after the ingestion and 30 to 45 minute after leaving the stomach (Van Weyenberg et al., 2006). The retention time of food in cecum is about 3 hours, to allow initial fermentation by microbes (Miyaji et al., 2008). The cecum is a blind sac, highly sacculated. At the top of the cecum, the ileo-cecal and the ceco-colic junctions (Fig. 3) are situated in relatively close proximity to each other, one through which digesta enters from the ileum and the other one through which the passage from the cecum to the right ventral colon (RVC) is facilitated. The cecum starts to contract 12 to 15 cm after the cecum-colic junction and traps digesta in the cecal base (Van Weyenberg et al., 2006). The next step, when the cecum is in a relaxed condition, includes some reflux, but most of the digesta move further into the right ventral colon. The reflux of excessive amounts of digesta is prevented by the sigmoid configuration of the junction. The barriers are the ileocaecal valve, the caecoventral colonic valve, the ventrodorsal colonic flexure (pelvic flexure), which separate the ventral from the dorsal colon and the dorsal small colonic junction, at which the digesta enter the small colon (Frape, 2004). After 3 hours the contraction forces the digesta through the valve to RVC (Van Weyenberg, 2006). The digesta is rich in cellulose and passes through the foregut with minimal degradation. Sugars and polysaccharides which bypassed enzymatic activity in the small intestine will be fermented in the cecum (de Fombelle 2003).

1.1.5.2 Colon

The colon is divided in four different parts: right ventral colon (RVC), left ventral colon (LVD), left dorsal colon (LDC) and right dorsal colon (RDC). Colon is about 3–4 m long in the adult horse, having a capacity of more than double that of the cecum. The diameter of the great colon varies considerably from region to region, but reaches a maximum in the right dorsal colon, where it forms a large sacculation with a diameter up

to 500 mm. The four parts of the great colon are connected by bends known as flexures. In sequence, these are the sternal, the pelvic and the diaphragmatic flexures (Frape, 2004). From the cecum, the digesta flows out via the cecocolical junction into the right ventral colon, which has a capacity about 29 – 32 litres (Varound et al. 2004) with a diameter 25-30 cm and a retention time of 3 hours (Miyaji et al. 2008). The proportion of cellulose in the digesta is decreased through the ventral and dorsal colon fermentation (de Fombelle, 2003). The contractions of the colon are complex. It is possible to identify three different contractions. The first type is a rhythmic contraction that moves in an aboral direction. The second type is also rhythmic, but propagates in an oral way. The third type of contractions is isolated and does not propagate in any direction (Van Weyenberg et al., 2006).

After the LVC, the digesta moves into LDC, then RDC, where further fermentation takes place before passing through the small colon, where water and remaining nutrients are absorbed prior to exiting the animal body via the feces (Dougal et al., 2012). It is important to highlight that the transition between LVC and LDC, volume 6-18 litres with a short retention time, more or less 1 hour (Varloud et al., 2004), is narrow, and this anatomic morphology is responsible of the selective retention of coarse particles (1 cm or more) in the cecum and in the ventral colon. This is the reason for which only the small particles and the liquid move on the LDC and RDC (Van Weyenberg et al., 2006). The RDC has a relatively high volume of 30-36 litres, a longer retention time, around 4 hours (Varloud et al., 2004), and a fairly low capacity to digest the fibre (Miyaji et al., 2008). All soluble carbohydrates and starch, which bypass digestion in the small intestine, pass through the cecum and reach the colon where metabolic diseases may originate as laminitis and acidosis (de Fombelle et al., 2003, Willing et al., 2009).

During the digesta passage, each region of the gastro-intestinal tract allows the mixing of digesta with GI secretions, hydrolysis by digestive enzymes, absorption of the resulting products, fermentation of resistant material by bacteria, fungi and archea and the absorption of the fermentation products. Rate passage through the equine gastrointestinal tract is best described by mean retention time (MRT) that is the

integrated average time between marker ingestion and excretion (Van Weyenberg et al., 2006). This is important because it provides indirect information on the extent to which feed is digested and fermented. The type of substrate that arrives in the hindgut is dependent of pre-cecal digestion, which in turn is related to the passage rate. Higher passage rates will induce a decrease in pre-cecal digestibility (Santos et al., 2011), the longer MRT increases the proportion of small particle size in the digesta and consequently increases fibre digestibility (Miyaji et al., 2011). In addition, retention times of digesta in the hindgut will affect digestibility, microbial activity and adsorption of water (Santos et al., 2011).

Miyaji et al. (2008) found that the MRT in the ventral colon, which is together with the cecum the major site of the fibre digestion, is the longest MRT (about 9 h) of all hindgut. In general, the rate of the feed particles movement is decreased when particle size rises. The circular and longitudinal muscle coats of the left ventral colon, pelvic flexure and left dorsal colon act together as a pacemaker, resulting in retropulsive-propulsive movements. The result of these movements is a physical separation of the particles larger than 1 cm, repulsed into the LVC, from the finer particles. These retropulsive movements keep the cecum and the LVC filled, imposing a delay time for fermentation of cellulose and for bacterial protein synthesis (Seller et al., 1982). The fluid is retained for prolonged periods in the RDC. From this last part of the colon the digesta pass dorsally into the colon transversum, where contractions of the muscular wall squeeze the bolus. The liquid then flows back (Fig. 4) into the proximal colon (Van Weyenberg et al., 2006).

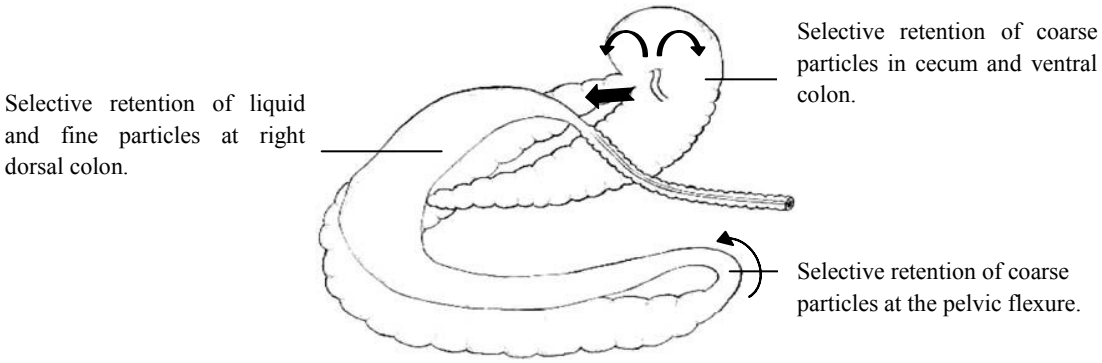

Figure 4 - Mechanism of separation of the liquid phase from particle in the equine hindgut.

It is important to highlight that each feed component has a maximum digestibility potential, because there is inert material completely resistant to digestion (lignin, cellulose and silica), but many other different factors can change the mean retention time. Drogoul et al. (2001) found that a large proportion of meal grain in the diet (50 of hay :50 of meal grain), reduce the retention time of barley in the small intestine with consequent increase of non-digested starch in the hindgut and, increased the possibility to have microbiological problem in this tract of gut. There are many different theories about the relationship between the body size and the MRT. Miraglia et al. (1992) affirm that the physiological state have an influence on MRT, in particular in the late pregnant mares this is 10h lower that of empty mares, regardless of feeding level. The exercise reduces the mean retention time and consequently also the enzymatic digestion (Pagan et al., 1998). Pearson and Merritt (1991) showed that the donkeys have a slower rate of passage of digesta through the gastrointestinal tract and, as a consequence, have a higher apparent digestibility of both organic matter and fibre fractions than that measured in ponies. In another study, where donkeys and ponies received molassed dehydrated alfalfa or oat straw, either *ad libitum* or restriction to about 70% *ad libitum*, and with a different composition, has been showen that donkeys had significantly higher apparent digestibilities for the main dietary components of the forages compared with those measured in ponies. The differences were more pronounced when a good quality alfalfa

diet was provided than when the poorer quality straw diet was fed. Another important aspect is the feed particle size. Drogoul et al. (2000) observed that fine particles (ground and pelleted) passing quickly through cecum, remained longer in the colon, causing an increasing of the lactic acid production and a pH value reduction (Medina et al. 2002).

1.2 End-products of microbial population activity in the gastrointestinal tract

As described in the previous paragraph, the retention time for digestion in each section of the GI tract depends on many different factors: diet, physiological conditions, physical conditions of the ingesta and microbiological activity. The health and welfare of the horse is influenced by all these factors and their interaction. The GI is the site where the hydrolysis occurs by digestive enzymes, followed by the absorption of the resulting products, and moreover by the fermentation activities of bacteria, fungi, and protozoa and the absorption of their end fermentation products.

The primary source of energy for hindgut fermenting herbivores is a wide range of carbohydrates. They can be divided into two groups: those hydrolysed and absorbed in the stomach and small intestine, i.e. monosaccharides, oligosaccharides and starch and those fermented in the hindgut, mainly fibre polysaccharides (cellulose and hemicellulose).

Compared with the large intestine, little information is available on the postprandial characteristics of the gastric ecosystem, but different experiments indicated abundant concentrations of active microbial populations in some prececal compartments, stomach and small intestine (de Fombelle, 2003, Varloud et al., 2007). These authors identified Lactobacilli, Streptococci and lactate-utilizing bacteria as the most representative population of these gastric compartments of horses. In the stomach, as a consequence of the production of gastric HCl secretion, the pH is acid. For these reason this microorganisms are considered to be acid-tolerant and, with α-*amylase* secreted by saliva, they hydrolyse the starch producing VFA, organic acid and lactate as final products. Different studies, as Alexander and Davis, 1963, Argenzio et al., 1974 and

Varloud et al., 2007, however proved that in the gastric content the concentration of VFA is low. The partition of digestion between the foregut and the hindgut as well as the factors of variation for diverse starches of the feeds or rations is little documented despite their importance in terms of nutrition and health for the performance horse. *Lactobacillus delbrueckii* and *Lactobacillus salivarius* have been identified in the GI content collected from slaughtered horses, that were previously fed a roughage-based diet and deprived of feed and water for 6 h before slaughtering (Al Jassim et al., 2005). Underlining that, in live, the stomach is emptying cyclically during the day, the ecosystem of the horse stomach changes during the first postprandial hour. The average postprandial pH (6.04) becomes more compatible with microbial development. The total anaerobic bacterial population is increased during the postprandial period (Varloud et al., 2007). In the same study Varloud et al. identified *Lactobacillus mucosae* as the species responsible to produce L-lactate under in vitro incubation, whereas all strains synthesized the D-isomer. The lactate, in feed rich of starch, mainly was converted in propionic acid (de Fombelle et al. 2003) whereas the glucose was converted in acetate.
Its concentration reached, alone, values about 11mmol/L of the total VFA (Varloud et al. 2007). Different reports (Nadeau et al., 2000, de Fombelle et al., 2003, Varloud et al., 2007) showed that VFA concentration increases after the meal and, in particular, mainly due to the production of acetate. For example Valourd et al. (2007) found that the acetate represents, on average, 80 mol-100 mol of the total VFA concentration and is increased by 10% within the first postprandial hour to reach 83 mol-100 mol of total VFA 2 h after the meal. The VFA could be absorbed through the gastric wall or the wall between the stomach and the small intestine. These VFA could be an important energy source for the mucosal cells. The glandular and pyloric mucosa absorbs VFA at pH 7.4, which can be observed in a postprandial state (Varloud et al., 2007).

In the stomach occurs also the microbial degradation of nitrogen-based compounds. In the stomach NH_3 derives from the hydrolysis of the urea by microbial urease (Varloud et al., 2007); but the main site of enzymatic hydrolysis of protein in the horse is the small intestine (Kern et al., 1974, Hymoller et al., 2012). Proteins digested in the small

intestine are absorbed as amino acids and contribute to the metabolic amino acids pool. The amount of protein hydrolysed in the small intestine is about three times higher than in the stomach, and the enzymes responsible for protein degradation are amino-peptidases and carboxypeptidases secreted by the wall of the small intestine (Frape, D. 2004). Microbial protein degradation is performed by proteolytic bacteria representing a high proportion of the total culturable bacteria, especially in duodenal samples. Counts of proteolytic bacteria per gram of sample were 3.0×10^6, 15.6×10^6 and 22.0×10^6 in the duodenal, jejunum and ileum, respectively (Mackie and Wilkins, 1988). The final products of the proteins degradation, ammonia and urea, are used by cecal bacteria as nitrogen source for their growth (Maczulak, et al. 1985).

All products, which escape the pre-cecal digestion, are degraded in the hindgut, where the greater VFA production caused by the anaerobic microorganism occurs. Mackie and Wilkins (1988) reported the molar proportions of acetate, propionate and butyrate in the hindgut 85:10:3, respectively. de Fombelle et al. (2003) identified the ventral colon as the site with the higher production of VFA. In the same report the quantification of bacteria in the different tracts of the horse intestine was analysed. The proportion of cellulolytic bacteria (with respect to total bacteria) was greater in the cecum than in the lower part of the hindgut (de Fombelle et al., 2003). In particular *Ruminococcus flavefaciens, Ruminococcus albu*s and *Fibrobacter spp.* were identified as the predominant cecal cellulolytic bacteria (Julliand et al., 1999). In other study the quantitative analysis of the intestinal microflora disclosed that the predominant bacterial population in the large intestine of horse (feed conventional grass-based diet) is represented by *Spirochaetaceae,* the *Cytophaga-Flexibacter-Bacteroides* assemblage, the *Eubacterium rectale-Clostridium coccoides* group, and unknown cluster C of the *Clostridiaceae.* Each of these groups represented 10 to 30% of the total microflora in each sampled horse. Among other bacteria, the following populations were found: the *Bacillus-Lactobacillus-Streptococcus* group, *Fibrobacter* and unknown cluster B, each representing 1-10% of the total microflora (Daly and Shirazi-Beechey, 2003). Members of *Spirochaetes* phylum have been shown to be major acetate producers using H_2/CO_2 as

substrate for acetogenesis in termites (Leadbetter et al., 1999) and similar function is supposed for *Spirochaetaceae* family in the equine colon responsible of the significant production of acetate for the horse gut (Santos et al., 2011). de Fombelle et al. (2003) found a greater concentration of lactobacilli and streptococci in the feces than in the other parts of hindgut, in particular, they showed a lower concentration of these bacteria in the cecum. The low concentration of streptococci and lactobacilli in cecum was explained by the lower amount of rapidly fermentable carbohydrates in this part of gut, which is in relation to the pelvic flexure and its capacity to separate small and big particles. On the other hand the major concentration of cellulolytic bacteria was found in the cecum and ventral colon. The opposite situation was found in the dorsal colon (de Fombelle et al., 2003).

1.3 Feeding of the horse

As previously said, the horses are free-ranging herbivores adapted to eat large quantities of high fiber feed and having a specialised gastrointestinal tract capable to ferment a wide range of plant species. Like all herbivores, the horses have developed strategies to select appropriate feed for their survival. This ability is very important for example to recognise toxic feed. Nowadays the horses are mainly used as a pet or livestock and, on the basis of several authors, the mismatch between the original and actual condition may have changed this ability, whereas the capacity to select a higher energy diet over a lower energy would seem unchanged (Cairns et al., 2002). Forages and concentrates used for horses feed are part of two families: *Gramineae* and *Leguminosae*. The main *Gramineae* used as forage are oat (*Avena sativa*), rye (*Lolium perenne*), barley (*Hordeum sativum*), fescues (*Festuca*), timothy (*Phleum pratense*) and cocksfoot (*Dactylis glomerata*), of which stems, leaves, flowers and seeds are used. The most used cereals seeds are those of oat, rye, barley, maize (*Zea mais*) and wheat (*Triticum aestivum*). The main *Leguminosae* used as forages are alfalfa-hay (*Medicago sativa*) and

clover-hay (*Trifolium pratense*), whereas the legumes seeds are those of pea (*Pisum sativum L.*), broad bean (*Vicia faba L.*), soy (*Glycine max L.*), carob (*Ceratonia siliqua L.*) (Martin-Rosset, 1994, Frape 2004). The international feed industry offers the greater diversity of products in the equine sectors. However, the concentrate fed to horses is rarely varied due to concerns over disturbances of gut microflora and potential onset of colic.

1.3.1 Forage

Forages are usually subdivided into fresh and dried (hays, silage and straw) and represent the primary feed source for horses. However, to meet the additional demands of energy for work, reproduction, lactation and growth, it is necessary to add supplements and concentrates. The limiting factor of digestibility of plant biomass food composed mainly of cellulose and hemicellulose is the amount of lignin, which cannot be decomposed by anaerobic microorganisms of hindgut. The lignin represent an important component of the cellular wall and its percentage is greater in mature forage and in the stems than in young forage, concentrate and leaves, with slim cellular wall easily digested. For this reason, the food digestibility is higher in the concentrate, about 90% in the corn and lesser in forages, about 75% in the fresh forage, (50-45% mature forage) and straw about 40% (Martin-Rosset, 1994).

Grass or fresh forage represents the total ration of horses at grassland. The chemical composition, the nutritional value and the digestibility of pasture is variable in function of the vegetative stage of the forage and of the botanical species. For example, the crude protein content may range from as little as 30 g/kg in very mature herbage to over 300 g/kg DM in young. The crude fibre content is related inversely to crude protein content, and may range from 200 to as much as 400 g/kg DM in very mature herbages.

The water-soluble carbohydrates of grasses include fructans and glucose, fructose, sucrose and raffinose. Their total concentration is very variable in function of the variety of forage and is greater in the stems than in the leaves. The highest concentration usually

occurs just prior to flowering. The cellulose content is generally within the range of 200-300 g/kg DM (dry matter) and that of hemicelluloses may vary from 100 to 300 g/kg DM. Like lignin, content of both of these polysaccharides increases with maturity. The proteins identify the main nitrogenous compounds in herbage and their total content is decreased with maturity, whereas the relative proportion of amino acids do not vary greatly.

The non-protein nitrogenous fraction of grass varies with physiological state and varies with species, variety and maturation stage. The main components of non-protein nitrogenous fraction are amino acids and amides.

Lipid content, that rarely exceeds 60g/kg DM, is represented mainly by triacylglycerols, glycolipids, phospholipids and sterols. Linolenic acid is the main fatty acid, comprising between 60 and 75% of the total fatty acids present, followed by linoleic and palmitic acids (McDonald, 1988).

The mineral content is variable, depending upon the species, stage and growth, but is very important that there is a good percentage of essential elements (Frape, 2004).

Green herbage is an important source of β-carotene, the precursor of vitamin A but, in small amount, is possible to find sources of vitamin D, that increases in the mature herbage, and vitamin E and K (McDonald, 1988, Frape 2004).

Hay or dried forage. The hay–making represents the traditional method of conserving of green crop. The aim of this method is to reduce the moisture content in the fresh crop to a level low enough to inhibit the action of plant and microbial enzymes. The moisture content in a green crop depends on many factors but may change between 650 to 850g/kg, tending to fall in mature plants. In the hay, the moisture content is between 150-200 g/kg (McDonald, 1988). So to preserve a good nutritional value, it is more important that the herbage is cut when plants have about 50% of spikes outside (gramineae) or when the flowers are germinated (leguminosae) (Martin-Rosset, 1994). This is the stage of maturity where the hay contains more DE (digestible energy), calcium, protein, β-carotene and some of the B vitamins, including folic acid (Frape,

2004). Hays made from legumes are generally richer in protein and mineral than grass hay, for example the value of lucerne hay consist in its relatively high content of crude protein, which may be as high as 200g/kg DM if it is made from a crop cut in the early bloom stage (McDonald, 1988). As previously said, the high fibre content extends the eating time with a higher level of chewing activity and saliva production, contributing to establish a correct environment in the pyloric region and in the hindgut, for microbial activity (Frape, 2004).

Silage is the material produced by the controlled fermentation of a crop of high moisture content. The fermentation is controlled either by encouraging the growth of lactic acid bacteria, which are present on the fresh herbage (native microflora), or by restricting fermentation by pre-wilting the crop or by using chemical additives. In both cases it is necessary to maintain anaerobic conditions. The nutritional value of the silage depends on the species, stage and growth of the crop and on the enzymatic microbial activity.

The bacteria capable to growth in anaerobic conditions are species as *Escherichia, Klebsiella, Bacillus, Clostridium, Streptoccocus, Leuconostoc, Lactobacillus* and *Pediococcus*. In function of the homo or heterofermentant activity, lactic acid bacteria produce different acids that are responsible for reduction of the pH value. *Escherichia* and *Klebsiella*, members of *Enterobacteriaceae*, are the major producers of acetic acid and they start the fermentation process because their pH optimum for the growth is about 7.0 Acidification consequently inhibits the growth of other bacteria and at about pH 3.8-4.0 microbial activity virtually ceases and the material remains stable for a long time, when anaerobic conditions are maintained (McDonald, 1988). A good silage for horses must have a minimum 25% of DM (Martin-Rosset, 1994).

Straw consists of the stems and leaves of plants after the removal of the ripe seeds by threshing, and are produced from cereal crops and from legumes. It is extremely fibrous product, rich in lignin and of extremely low nutritive value, about 0.26 to 0.33 UFC v/kg DM (Horse Feed Unit). Oat and barley straw have 10-15% more of energetic value than rye and wheat straw. The straw lacks some important compounds as phosphorus, calcium and vitamin A. Horses eat less straw than hay (about 1.0-1.8 kg in respect to

1.5-3.0 of DM/100kg live weight) due to its less digestibility caused by high lignin content. Straw stays in the hindgut for long time and can induce colic (Martin-Rosset, 1994).

1.3.2 Concentrate: cereals and legumes

The name *"cereal"* is given to those members of the Gramineae which are cultivated for their seeds. They are essentially carbohydrate concentrates, the main component of the dry matter being starch, about 40-75% of DM (Martin-Rosset, 1994). The dry matter content, in normal storage conditions, is generally within the range of 850-900 g/kg.

85-90% of the nitrogenous components are in form of protein. The total content of protein in the grain is variable; expressed as crude protein, it normally ranges from 80 to 120g/kg DM, although some cultivars of wheat can contain as much as 220g/kg DM. They are deficient in lysine, threonine, methionine and in Ca (less than 1 g/kg DM), but have a good percentage of P, about 3-5 g/kg DM of phytic acid (McDonald, 1988). The ratio Ca/P is variable for the different grains (from 0.10 to 0.25) but optimally is lesser than 1.5 (Martin-Rosset, 1994). The cereals are all deficient of vitamin D, but they are good source of vitamin E and thiamine (McDonald, 1988).

The lipid content of cereal grains varies with species. For example, wheat, barley and rice contain 10-30 g/kg DM, sorghum 30-40 g/kg and oat 40-60g/kg DM (McDonald, 1988). The oil content of cereal grains varies from about 15 to 50 g/kg, with oats containing slightly more than maize, which in turn contains more than barley or wheat. Oil is rich in polyunsaturated fatty acids, of which the principal one is linoleic acid which generally constitutes about half the fatty acid composition (Frape, 2004).

<u>*Cereal by-products*</u> can originate from milling, brewing or distilling industries but primarily from pearl barley, maize, oat industry, and milling wheat. They have a good amount of crude proteins, so they can be used as supplements in the diet (McDonald, 1988).

Legumes have a high percentage of protein, about 20-40% DM, and fats (Bonciarelli, 2001), but have a low content of Ca and Mg (Martin-Rosset, 1994). Usually they are supplied with cereals.

Protein concentrate contains all vegetable proteins edible for farm animals. Oil-seed residues, peas, beans, yeast, soya beans, linseed, cottonseed and sunflower seed are used for horses and ponies. These feedstuffs, in general, contain higher percentage of proteins than cereals grain. Oil-seed meals are much richer sources of protein and their balance of amino acids is superior. They have a good amount of B vitamins and phosphorus, but are deficient of calcium. However, linseed meal is a poorer source of lysine than soya, considering the best quality of these proteins. Sunflower seeds are rich in the sulphur amino acids, cystine and methionine, although in horse diet these amino acids are rarely limited (Frape, 2004).

The intake rate and eating behaviour is affected by feed processing because the treatment application changes the physical and chemical composition of grains. There are two basic types of processing providing a better chemical stability of the cereal grain: the "*hot processes*" and the "*cold processes*". The hot processes include steam-flaking, micronisation, extrusion and hot pelleting. Cereals should be cooked only in the presence of water in order to minimize the risk of heat damage to proteins and oil. Steam flaking is often applied on maize and, it caused slightly higher digestibility than unprocessed grain. Steaming and flaking is also known to increase the proportion of propionate acid in the rumen of herbivores (McDonald, 1988).

The term micronisation is used to describe cooking by infrared radiation energy before flaking to produce micronized grains. Water is added before micronisation and the temperature ranging from 150°C to 180°c for 30-70s is applied (Frape, 2004). In this process the starch granules swell, fracture and gelatinise thus making them more available to enzymes attack in the digestive tract (McDonald, 1988).

The extrusion processes use temperature around 120°C for about a minute, and like the steam, have been shown to improve the digestibility of dry matter, organic matter, starch

and the nitrogenfree extract of cereals and nuts without interfering with the digestibility of crude protein (Frape, 2004).

The *cold processes* include grinding, rolling, cracking or crimping, cold pelleting and addition of organic acid or alkalis. These are used as processing feed for ruminants, pigs and poultry (McDonald, 1988).

The importance of diet composition and delivery format is kwon for long time. In 1966 Hintz and Loy, noted that the voluntary intake of pelleted or not-pelleted ration changed the rate passage of digesta, which was apparently faster for the pelleted ration. The pelleted ration was consumed in a significantly shorter period of time. The fresh feces of horses fed with the nonpelleted ration contained a significantly higher percentage of dry matter than the feces of horses fed with the pelleted ration. These results are supported also by Cuddeford et al. (1992). Rosenfend and Austbo (2009b) compared different forms of barley, maize and wheat, all ground (1 mm screen), pelleted (ground 1 mm screen and after processed at 82-85°C to produce 8 mm pellets), estruse (ground 1 mm screen and after processed at 130°C to produce 13 mm pellets) and micronized (with 8-10% of water and coke with infrared radiation for 45 s at 112-115°C before flaking). Their results showed that maize have a longer retention time in the cecum than other grains, and the ground grains have a longer MRT in the hindgut, but, the total mean retention time (all gastrointestinal tract) is longer for pelleted grains. Heat treatment may increase the solubility of fibers, due to increased capacity of water-binding (Vranjes and Wenk, 1995). However, the estruded grains contained less fat compared with the other treatment process. In general high-temperature treatments have consequences on the physical and chemical properties of the grains, increasing the digestibility of starch and proteins but, many external factors can change all parameters (Rosenfend and Austbo, 2009b). It is important to highlight that a higher disponibility of starch can modify the microbial population. An increase of Lactobacilli and Streptoccoci, and a decrease of cellulolytic bacteria was described in the hindgut of horses fed with a starch rich diet.

1.4 Influence of the feed and the management on the intestinal welfare of horse: acidosis, laminitis and colic

The feed management procedures have an important implication on the utilization of nutrients from both concentrate and forage components of the ration and, consequently on the health and welfare of horses. Several studies described the different impact of the forage:concentrate ratio on the digestibility and intestinal microbiota. A diet rich in readily fermentable carbohydrates is now often use with the aim to meet the increased energy requirements of the modern performance horse.

Daly et al. (2012) determined the alterations in microbiota and their fermentation products in the horse hindgut in response to dietary variations (grass-only diet and concentrate diet) and described the possible consequent intestinal diseases. In horses fed with concentrate the abundance of *Lachnospiraceae, Bacteroidetes* (fibrolytic and saccharolytic bacteria) and *Bacillus-Lactobacillus-Streptococcus (BL*S) group was found. Member of this group, *Streptococcus bovis,* is implicated in the aetiology of lactic acidosis both in horses and in the ruminants (Herrera et al. 2009). In the same study (Daly et al. 2012) a lower abundance of *Fibrobacter* group was observed with consequent reduction on the fiber digestion, whereas, the percentage of lactate-using bacteria, as *Veillonellaceaea* (cluster of Clostridiaceae), did not change (Daly et al., 2012). However, different authors observed different results. Goodson et al. (1988) did not confirmed the decrease of cellulolytic bacteria due to the diet, but he described the protozoan population decrease after abrupt change, from hay to concentrate and persistence of three genera throughout the concentrate feeding period. These results have not been confirmed by Moore and Dehority (1993), who described no effect of diet rich with forage or rich in concentrate on the cellulolytic bacteria and fungal and protozoal population. Nevertheless, it is clear that diet has influence on digestion. The administration of starch rich diet decreases the acetate:propionate ratio, increases the level of blood lactate, and consequently reduces the pH in the hindgut (Willard et al., 1977, Stevens and Hume, 1998, Muhonen et al., 2009). These studies showed that different diets have important influence on the equine intestinal microbiota and,

consequently, on the intestinal disease. Different diets are correlated with alterations in intestinal microbial ecosystems and production of microbial fermentation products (such lactic acid), thus creating the predisposition of the host to intestinal disease as acidosis, laminitis and colic.

Acidosis is physiologic state induced by consumption of large quantities of rapidly fermentable carbohydrates in form of starch or sugar. In horses it is characterised by elevated lactic acid levels primarily in the cecum and colon and later, in the blood with the consequently lower pH. As previously mentioned, several authors (de Fombelle et al., 2003, Varloud et al., 2007, Daly et al. 2012) found an increase in number of *Lactobacilli* and *Streptococci*, the bacteria responsible for production of lactic acid in horses fed with high-starch diets. Rowe et al. (1994) showed that, horses fed with high concentrate diet, have a higher blood levels of lactic acid, lower fecal pH, lower fecal solidity and greater tendency to develop laminitis. A different situation was found in horses fed with high-energy forage-only, where the microbial population composition was more stable and with a lower count of *Streptococcus bovis/equinus* and Clostridiaceae cluster (Willing et al. 2009).

Laminitis is the painful and debilitating disorder resulting in cellular damage and inflammation of the tissues, comprising the bonds supporting the pedal bone within the hoof capsule. Gastrointestinal disturbs are the only known reason of this pathology. Several studies showed the relationship between alimentary carbohydrate overload and laminitis in horses. For horses suffering with laminitis was typical the increase of Gram-positive bacteria and the decrease of Gram-negative population in the hindgut (de Fombelle et al., 2003, Milinovich et al., 2006, Varloud et al., 2007, Willing et al., 2009, Daly et al. 2012), which is probably connected with a high production of amine (greater than 1 microM), which is responsible for vasoconstriction problems, if is released into the blood from the gastrointestinal tract (Bailey et al. 2003). In particular, was found that the caecal concentrations of phenylethylamine, isoamylamine, cadaverine, diaminoheptane and spermidine were significantly higher in horses on spring/summer

grass compared with those on winter grass or hay. Elliott and Bailey (2006) explained these changes by higher amount of microorganisms producing amino acid decarboxylase enzyme capable to convert the free amino acids into monoamines. Dates of Bailey (2003) showed that many amines are present in the equine hindgut, some of which may have the potential to cause peripheral vasoconstriction if released into the blood circulation from the gastrointestinal tract. The main theory for the pathogenesis of acute laminitis involves the development of vasoconstriction within the foot as a consequence of high grass diet inducing the onset of laminitis in the horse (Milinovich et al., 2006, Elliott and Bailey, 2006).

Colic is a typical expression used for different type of the gastrointestinal pain in horses. The following circumstances have been identified as risk factors for horse colic: feeding practices (type and quality of food, type and changes of feeding), the intrinsic factors of horses (sex, age and breed), management (type and changes of housing and activity), medical history and parasite control.

Many colics is explained by the presence, of a thick, sticky mass of fermenting feed or a compacted mass of roughage in the stomach, or intestine (Frape, 2004). Low quality of forage and concentrate can be the risk factors for colic. Cohen et al. (1999) showed that a high percentage of fibre and hay of poor quality in the diet increases the probability of impaction and predisposing horses to colic. Hay of poorer quality is often less digestible and can cause alterations in colonic pH, VFA production and, even in the colonic microflora inducing the disorders of intestinal function resulting in colic. The risk of colic was multiplied by 2 during the 2-week period when the changes in feeding were performed (Cohen et al., 1995). Similarly a rich concentrate diet, as previously said, induced a depression of cellulolytic bacteria in the colon and increased the concentration of lactate-utilizing bacteria, lactobacilli and streptococci. These changes of the microflora are associated with a significant decrease of intestinal pH and VFA ratio, an increase of lactate concentration, and consequent increased risk of this intestinal disease. (Julliand et al, 2001).

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CHAPTER 2

GASTROINTESTINAL MICROBIAL COMMUNITY OF HERBIVORES

The importance of the microbial ecology and diversity of microorganisms in the digestive tract of herbivora has gained increasing attention in response to recent trends in the global livestock production. The microorganisms in the digestive tracts of ruminants and non-ruminant herbivora have a profound influence on the conversion of feed into end-products, which can impact on the animals and environment. Especially with respect to the livestock sector there is an increasing need to understand these processes for better management and use of both the feed-base and other natural resources which underpin the development of sustainable feeding systems. The microbial ecology of the gastro-intestinal tract has developed as a specialized research field in microbiology and focuses on investigation about the abundance and diversity of the organisms, their relationship with the others and the host animal. Until recently, knowledge of gut microbiology was primarily obtained using classical culture based techniques, such as isolation, enumeration and nutritional characterization, which probably only account for 10 to 20 percent of the microbial population. Now, the new nucleic acid-based technologies, which are culture independent, can be employed to examine microbial ecology and diversity. These technologies have the potential to revolutionize the understanding of gut microbes function and overcome the limitations of classical based techniques, including isolation and taxonomic identification of strain, which are important for an efficient digestive function and better understanding of the microorganisms roles in relation to achieve high productivity and decrease environmental pollutants and contamination of the food chain.

The digestive tract of herbivora is inhabited by a high density of resident microbiota, consisting of bacteria, protozoa, archaea and fungi, which degrade the ingested plant materials. This complex, mixed, microbial culture forms a closely integrated ecological unit among themselves and the host animal, as well as playing a vital role in the nutritional, physiological, immunological and protective functions of the host. The development of the microbial populations in the digestive tract of higher animals

commences soon after birth and involves a complex process of microbial succession and many microbial – host interactions which, eventually resulting in dense, stable microbial populations inhabiting characteristic regions of the gut. The rumen is one of the most extensively studied and well-documented gut ecosystem because of the importance of ruminants (cattle, sheep, goats, camels and yak), but the increased attention is devoted also to the microbiome of non-ruminant herbovires as for example horses with the respect to their growing sport activity.

The general present knowledge about the diversity of the gut microbes is summarized in this chapter with special attention to the microbial composition of the horse digestive tract and enlarged paragraphs dealing with anaerobic gut fungi.

2.1 Bacteria

Bacteria are the most abundant and diverse part of gut microbiome. They ferment and degrade the plant fibers in a coordinated and complex manner which results in the conversion of plant materials into digestible compounds for the host animal, such as volatile fatty acids (Fig.1) and bacterial proteins.

The bacterial sequences generated from herbivoral gut samples can be assigned in 19 existing phyla and 180 genera, of which the *Firmicutes*, *Bacteroidetes* and *Proteobacteria* are the most predominant phyla.

More than 90 percent of the *Firmicutes* sequences is assigned to genera within the class *Clostridia* while *Streptococci* are prominent within the class *Bacilli*. Within the *Clostridia*, *Lachnospiraceae, Ruminococcaceae* and *Veillonellaceae* are the largest families. The predominant genera including *Butyrivibrio, Acetivibrio*, *Ruminococcus*, *Succiniclasticum*, *Pseudobutyrivibrio* and *Mogibacterium*. In the *Bacteroidetes* phylum the majority of the sequences is assigned to the class *Bacteroidia* and *Prevotella* is the most predominant genus. *Prevotella* is generally in high abundance in the rumen. *Proteobacteria* are represented by the *Gamma proteobacteria,* being the dominant class.

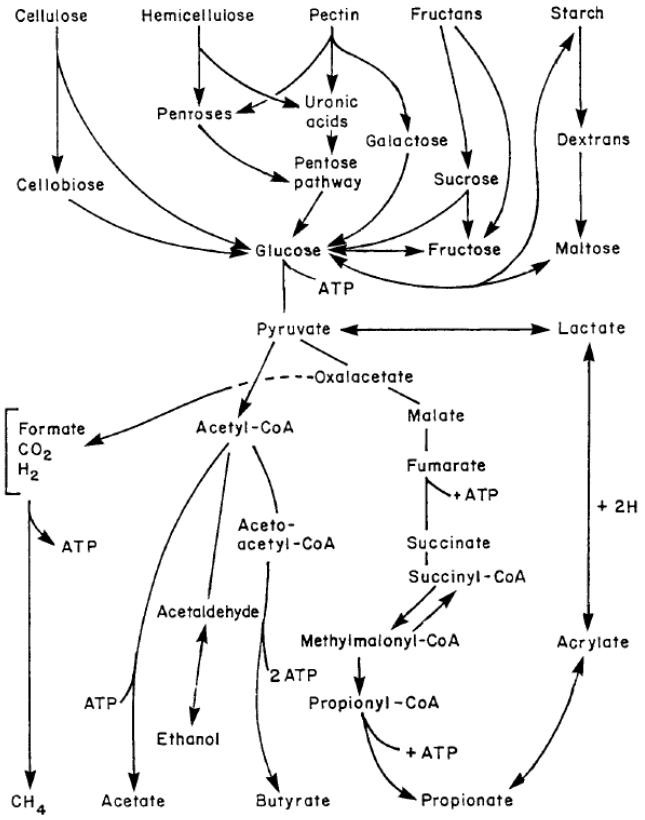

Figure 1 - Carbohydrate metabolism (Van Soest 1994).

Cellulose, hemicellulose (structural polysaccharides) and pectin of the plants cell wall, fructans and starch (storage polysaccharides) are ingested with the diet and fermented by the microbial community in absence of O_2 into oligosaccharides and monosaccharide, which are converted into pyruvate, that is reduced anaerobically to short-chain fatty acids (SCFA), CO_2, CH_4.

2.1.1 Bacteria of the horse digestive tract

Horse's intestinal bacteria can be classified into cellulolytic, hemicellulolytic, amylolytic, proteolytic, lactate-using and glycolytic bacteria. Daly et al. (2012) showed that the majority of sequences recovered from the horse digestive tract samples are phylogenetically affiliated to several known groups of anaerobic bacteria, namely to low percentage G+C Gram positive bacteria, belonging to the phyla *Firmicutes* and

Bacteroidetes. *Firmicutes* are represented specially by the bacterial order Clostridiales, family *Lachnospiraceae*, which is an important group of fibrolytic and saccharolytic bacteria including *Clostridium spp., Butyrivibrio spp., Ruminococcus spp.* and *Eubacterium spp*. Phylum *Bacteroidetes* is represented mostly by *Bacteroides* and *Prevotella spp*. (saccharolytic cluster). Julliand et al. (1999) and Hastie et al., (2008) suggested that the cellulolytic bacteria of the hindgut of the Equidae are represented mainly by strains of *Ruminococcus flavefaciens*, *Ruminococcus albus* and *Fibrobacter succinogenes,* which are important bacteria active in the fibre breakdown.

Kobayashi et al. (2008) described in the hindgut of horses bacterial species of *Bacillus, Lactobacillus and Streptococcus*, which are groups of bacteria not able to ferment structural carbohydrates directly but they are able only to utilise oligosaccharides and soluble carbohydrate released by fibrolytic organisms (Daly et al., 2012). *Streptococcus bovis* is capable to degrade starch, originating lactate as the main fermentation product.

S. bovis was found to be associated with lactic acidosis phenomenons in the rumen and in the hindgut of horses (Rowe et al., 1994, Goad et al., 1998). Increased levels of lactic acid cause a rapid decline in the intestinal luminal pH and reduced the rate of butyrate transport by > 50% (Ritzhaupt et al., 1998), thus causing digestive disorders.

Shepherd et al. (2012) described by molecular techniques the presence of *Actinobacillus* spp. and *Succinovibrio* spp. These bacteria are supposed to be involved in the enzymatic hydrolysis of protein, probably in small intestine, which is the main site where the enzymatic hydrolysis of protein occurs in the horses digestive tract. However, the vast majority of bacteria from the equine large intestine (89%) does not correspond to any recorded sequences, which means that anaerobic bacteria in the horse hindgut are mainly unknown and represent many novel bacterial species (Daly et al. 2001)

2.2 Protozoa

Protozoa are eukaryotic unicellular organisms which live as single individuals or in simple colonies. They emerged as an evolutionary group more than 10^9 years ago, separating from the eukaryotic line that led to plants and animals before the fungi appeared (Elwood et al. 1985). They are divided in five different phyla: Mastigophora, Sarcodina, Ciliophora, Opalinida and Sporozoa (Curtis and Barnes, 1996). Protozoa found in the herbivorous intestine are members of the Ciliophora phylum. This phylum is characterized by the presence of cilia (length 5-20 μm) used for swimming or crawling in the fluid. The ciliate protozoa are capable to ferment carbohydrates by the cellulolytic activity, to degrade pectin substances by pectin lyase and pectinesterase enzymes (Bohomme-Florentin, 1988). Activities of amylase, xylanase and urease have also been described for protozoa (Santra and Karim, 2002). Protozoa play a major role in the ingestion of particulate protein, including plant (supplementary) protein and a lesser role in uptake of soluble protein, peptides and amino acids. Protozoa have a protease activity similar to that of bacteria and rapidly deaminate amino acids.

Entodinium is the dominant genus in the rumen (Sylvester et al., 2004), but is considerable protozoal diversity representing many different genera and species, is found in different herbivores and is dependent on the diet (Kittelmann et al., 2013). Major protozoal intestinal species are *Epidinium caudatum*, *Entodinium caudatum*, and *Isotrica prostoma* (Tymensen et al*.,* 2012). The following genera are also common in the gastrointestinal tract of herbivores and their amount and proportion depends on the diet: *Isotricha*, *Dasytricha*, *Ostracodinium*, *Diplodinium*, and *Diploplastron* (Tymensen et al., 2012).

2.2.1 Protozoa of the horse digestive tract

The first studies about ciliate protozoa from horse intestine appeared long time ago (Gruby, D. 1843; Fiorentini, 1890; Budle, 1895; Gassovky, 1919 and Hsiung, 1930) and the horse protozoa were intensively studied, however as parazites. Their endosymbiotic role was discovered later. By cultivation method, five ciliate species from the cecum and colon of horses have been described: *Cycloposthium edentatum*, *Cycloposthium ishikawai*, *Tripalmaria dogieli*, *Cochliatoxum periachtum*, and *Paraisotricha colpoidea* (Strueder-Kypke et al., 2007). At the present, 72 species of protozoa, primarily ciliates, have been described as normal inhabitants of the equine large intestine, with some tendency to species differences between compartments (Frape, 2004). The horse protozoa differ substantially from rumen cilliates, indicating the influence of the host animal. Species the following genera have been described in the horse digestive tract: Cycloposthium, Spirodinium, and Paraisotricha, Triadinium and Blepharocorys (Snelling et al., 2011, Gocmen et al., 2012).

Adam K., in 1951, analysed the ciliate population in the intestine of the horse found that the dorsal colon has the major concentration of this microorganism and moreover there is a different ciliate population in the first tract (ventral colon) and in the second tract of the hindgut. It is supposed that this change is a consequence of the presence of the pelvic flexure (Adam, 1951). This author also found that the amount of protozoa in the gastrointestinal tract changes in relation to the diet. In another report, the ciliate population belonging to thirty four species and 21 genera were identified comparing the feces samples from horses, mules and donkeys living in the same area (Gurelli, 2012). In this report, the same species found by Adam (1953), in particular *Bundleia postciliata, B. angusta and B. microcorys*, were identified in all horses. *Cycloposthium spp*. and *Blepharocorys spp.* possess β-galactosidasic and lipolytic activities. They are able to degrade the pectine substances and are involved in the degradation of hemicellulosic substrate (xylan endo-1,3-β-xylosidase activity) from cell-wall (Bonhomme-Florentin, 1988).

2.3 Archaea

In 1990 Woese et al. proposed a subdivision of the domain of Archaea in two kingdoms: *Euryarchaeota,* encompassing the methanogens and their phenotypically diverse relatives and *Crenarchaeota,* comprising the relatively tight clustering of extremely thermophilic archaebacteria. Archaea of the herbivorous digestive tract are strictly anaerobic methanogens, which means that they belong to *Euryarchaeota.* They are strictly anaerobes, obtaining energy for growth by the oxidation of H_2 and sometimes formate or CO_2 or both with the reduction of CO_2 to methane (Balch et al., 1979). *Methanogens* can use, as substrate, CO_2, H_2, formate or acetate directly produced by fermentative bacteria, fungi and protozoa (Garcia, 2000). The end product of their activity is CH_4. Methanogens have been classified into 5 orders (Methanobacteriales, Methanococcales, Methanomicrobiales, Methanosarcinales, and Methanopyrales), 28 genera and 113 species (Garrity et al., 2007, Liu and Whitman, 2008), but surprisingly only few of them have been isolated from the rumen, where methanogens are well studied with respect to global warming problems (Johnson and Johnson, 1995).

Based on the analysis of global data sets available in public databases, the majority (>90%) of the rumen archaea are affiliated with genera *Methanobrevibacter* (> 60%), *Methanomicrobium* (~15%) and a group of uncultured rumen archaea (~16%) commonly referred to as rumen cluster C (Janssen and Kirs, 2008; St-Pierre and Wright, 2012). Within the genus *Methanobrevibacter*, two major clades *M. gottschalkii* (contains *M. gottschalkii*, *M. thaueri*, and *M. millerae*) and *M. ruminantium* (*M. ruminantium* and *M. olleyae*) dominant in the rumen. Members of other groups of methanogens, including *Methanimicrococcus spp.*, *Methanosphaera spp.*, and *Methanobacterium spp.* appear in low abundance.

2.3.1 Archaea of the horse digestive tract

Only limited studies have characterised archaea in the horse digestive tract. Lin and Miller (1998) described the sequences of DNA from horse feces as *Methanobrevibacter smithii.* Jensen (1996) also described species of Methanobrevibacterium as the predominant methanogen in horse feces. The number of studies of methanogenic bacteria present in monogastric animals is limited. Methane production by monogastric animals is lower than that on ruminants. However, methane production by large herbivorous monogastric animals, such as horses, mules and asses, is substantial up to 80 l per animal per day. On the other hand, in the work of Yamano et al. (2008) archaea were detected only for native horses, but not in light horses (Yamano et al., 2008) Limited results indicate that methanogens of horse gastrointestinal tract need to be studied in more detail.

2.4 Anaerobic Fungi

Gut fungi are an unusual group of symbiotic zoosporic fungi occupying an unique ecological niche, the anaerobic environment of the rumen and gastrointestinal tract of large herbivores. The original notion "rumen fungi" has been replaced by term "gut fungi" after the discovery of their incidence in the hind-gut of animals like horses, rhinoceros or elephants. In the digestive tract, fungi contribute, in association with bacteria and protozoa, to the hydrolyzation of diet fiber resulting in end fermentation products (volatile fatty acids) which can be utilized by the host animal as nutritive source. The contribution of the gut fungi to cell wall degradation has not been yet exactly determined, however some studies suggested that fungi may greatly exceed the fermentation part of bacteria (Lee et al., 2000). Unique traits of the gut fungi specially anaerobiosis, possession of hydrogenosomes instead of mitochondria, multiple flagella and extremely low genomic GC content make these microorganisms extraordinary in the fungal kingdom. Despite intensive research, there are still many questions regarding

morphology, genetics, enzymology, taxonomy, diversity, and importance of gut anaerobic fungi for host animals.

2.4.1 Taxonomy

Zoospores of gut fungi observed for the first time in the rumen fluid more than one hundred years ago, were originally considered as protozoans. Only C. Orpin in 1975 recognized these cells as motile life stage of fungi and classified them as Phycomycetes. Description of chitin in their cell walls (Orpin, 1977a) however proved that these organisms are true fungi, despite their strict anaerobiosis, and in 1980 the anaerobic fungi were assigned to the class Chytridiomycetes (Barr 1980, 1988). Further advance in systematic of gut fungi was driven by modern methods of molecular biology. Multi-gene dataset phylogeny of DNA sequences of ribosomal RNA operon (18S rRNA, 28S rRNA, ITS) combined with protein coding genes (EF1α, RNA polymerase II subunits RPB1and RPB2) led finally to the separation of anaerobic fungi from Chytridiomycetes and to the formation of the new phylum Neocallimastigomycota including one class Neocallimastigomycetes, one order Neocallimastigales and a single family Neocallimastigaceae (Tab.1 Hibbett et al., 2007). Inside the family six genera of anaerobic fungi including *Neocallimastix*, *Piromyces*, *Caecomyces*, *Orpinomyces*, *Anaeromyces* and *Cyllamyces* have been determined up to now. The table 2 (Griffin et al., 2009 and Ho, 1995) shows all known cultivable species, however, recent research dealing with diversity of animal gut fungi indicates that the number of 6 genera and 21 species of anaerobic fungi is not final.

Table 1 - Classification of gut fungi. (Hibbet et al, 2007).

Domain	Eukarya
Fingdom	Fungi
Phylum	Neocallimastigomycota
Class	Neocallimastigomycetes
Order	Neocallimastigales
Family	Neocallimastigaceae

Table 2 - List of known cultivable strain of anaerobic fungi (Griffin, G.W. et al. 2009 and Ho Y.W. Barr D.J.S 1995).

Genus	**Species**
Neocallimastix	*frontalis*
Neocallimastix	*hurleyensis*
Neocallimastix	*patriciarum*
Neocallimastix	*variabilis*
Anaeromyces	*elegans*
Anaeromyces	*mucronatus*
Caecomyces	*communis*
Caecomyces	*equi*
Caecomyces	*sympodialis*
Cyllamyces	*aberensis*
Orpinomyces	*bovis*
Orpinomyces	*intercalaris*
Orpinomyces	*joyonii*
Piromyces	*citronii*
Piromyces	*communis*
Piromyces	*dumbonica*
Piromyces	*mae*
Piromyces	*minutus*
Piromyces	*polycephalus*
Piromyces	*rhizinflata*
Piromyces	*spiralis*

Neocallimastix, *Caecomyces* and *Piromyces* represent the monocentric genera (nuclei are concentrated inside sporangia, see figure 2) whereas *Anaeromyces, Cyllamyes* and *Orpinomyces* were identified as the polycentric genera (nuclei are spread inside the whole mycelium, see figure 3). Anaerobic fungi differ considerably from all known members of the Fungi Kingdom in many characteristics. The most apparent exceptional feature is the anaerobiosis. Rumen or gut fungi are the only fungi that do not only need any oxygen for their life cycle, but oxygen is toxic for them. Anaerobiosis implicates that, gut fungi cannot "breathe" and therefore do not require mitochondria, which are replaced by hydrogenosomes.

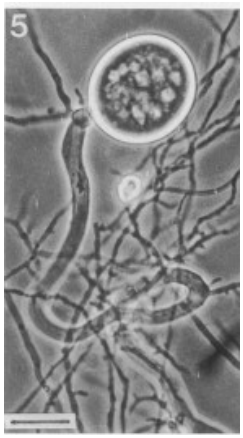

Figure 2 - A monocentric thallus with a single sporangium (*N. frontalis*). Ho et al., 1995

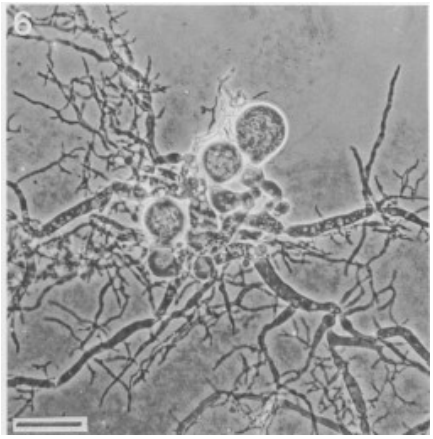

Figure 3 - A polycentric thallus with a many sporangia (*O. joyonii*). Ho et al., 1995

2.4.2 Life cycle and zoosporogenesis

The phase of the life cycle of anaerobic fungi alternates between a motile zoospore stage and nonmotile vegetative stage. The zoospores actively move by flagella, whose numbers differ in relation with the genera. Zoospores are attracted to freshly ingested plant tissues, presumably by chemotactic response to soluble sugars (Orpin and Bountiff, 1978) and/or phenolic acids (Wubah and Kim 1996). Released zoospores attach to the feed particles, encyst and germinate to produce fungal thallus composed of the rhizoids and the sporangium. The rhizoids can be highly branched as in *Orpinomyces*,

Anaeromyces, *Neocallimastix* and *Piromyces* spp. or may consist of spherical or ovoid bodies (holdfast or haustoria) as in *Caecomyces* and *Cyllamyces*. The rhizoidal system penetrates the plant tissue and the sporangia develop on the exterior surface of the plant fragment or within hollow stems. Two type of zoosporangial development can occur in anaerobic fungi. Endogenous development, where the nucleus is retained in the encysted zoospore, which enlarges into a zoosporangium, or exogenous development, where the nucleus migrates out of the zoospore and the zoosporangium is formed in the germ tube or sporangiophore (Barr et al., 1989, Ho et al., 1993). The concentration of nuclear material in a zoosporangium is typical for monocentric species (*Neocallimastix, Piromyces, Caecomyces*), while nuclear migration throughout the hyphal mass is typical for polycentric species (*Anaeromyces, Orpinomyces, Cyllamyces*) (Gaillard et al. 1989). Mature zoosporangia release zoospores and life cycle is reiterated. Zoosporogenesis can occur as early as 8h after germination under appropriate conditions (Orpin 1977a,b, France et al., 1990, Theodorou et al., 1993) but normally life cycle of anaerobic fungi varies between 24 – 32h (Lowe et al., 1987b, Wubah et al., 1991). The life cycle of gut fungi probably has a resting stage providing their survival for long periods of dessication and exposure to oxygen. The first indication of this ability is the presence of anaerobic fungi out of the rumen, in saliva and feces (Lowe et al., 1987a) and the survival of gut fungi in dried feces (Milne et al., 1989, Davies et al., 1993). This oxygen-resistant dormant stage is supposed to be important in inter-animal transfer of fungi via accidental or intentional coprophagy in addition to transfer through salivary contact (Lowe et al., 1987a).

2.4.3 Carbohydrate fermentation and end fermentation products

Anaerobic fungi recover energy from the fermentatin of carbohydrates. The common plant mono- and disaccharides (fructose, glucose, sucrose, xylose, cellobiose and gentiobiose etc.) are used by all isolates. Strains differences are evident in the utilization of maltose, galactose and mannose, whereas L-arabinose is not fermented (Gordon and Phillips, 1998).

Concerning storage polysaccharides, most anaerobic fungi utilize starch and glycogen, but some strains of *Caecomyces*, *Piromyces* and *Anaeromyces* do not ferment neither these polymers nor their component maltose (Phillips and Gordon 1988, 1995). Anaerobic fungi grown on glucose as carbon source produce the major end-products, acetate, formate and ethanol, variable amounts of lactate and malate and small amount of succinate. All isolates also produce CO_2 and H_2. The relative amounts of short chain fatty acids differ among the genera. For example the *Orpinomyces* sp. forms large proportions of formate, acetate and ethanol, with lesser amounts of lactate and malate, while the *Anaeromyces* sp. forms substantial amounts of lactate and malate, but smaller proportions of formate, acetate, and ethanol (Phillips and Gordon 1995). Some isolates of *Piromyces*, especially equine strains, do not produce any lactate (Ho et al., 1996, Julliand et al., 1997). Moniello et al. (1996) found that, in the *Neocallimastix frontalis RE1*, some plant secondary metabolites (coumarin and spartein) can strongly inibit the cellulose degradation capacity by endoglucanase enzyme.

From this, we can realize how, the cellulolytic activity of these fungi, can be inhibited by some components of the diet and, probably improved from others.

Anaerobic fungi use only the Embden-Meyerhof-Parnas pathway (glycolysis) for the glucose catabolism to pyruvate or phosphoenolpyruvate (O'Fallon et al., 1991, Reymond et al., 1992, Durand et al., 1995). Pyruvate is then converted into end fermentation products mentioned above in the cytoplasm and the malate is directed into hydrogenosomes, where the end products are acetate, CO_2 and H_2. Detailed process is described in the paragraph 2.4.4.

2.4.4 Hydrogenosomes

The energetic centre of anaerobic fungi is represented by hydrogenosomes. These organelles (Fig. 4), found in a variety of Protists but only in this phylum of Fungi (Neocallimastigomycota). Hydrogenosomes decarboxylate, under anoxic conditions, malate into acetate, CO_2, and H_2 with concomitant production of energy as ATP (Müller, 1993). In anaerobic fungi the malate is dehydrogenated to pyruvate by the malic

enzyme. This interconversion from malate to pyruvate is however not present in ciliate hydrogenosomes, where pyruvate is the first substratum. The metabolic way of pyruvate in hydrogenosomes and mitochondria is compared in figure 4. ATP is produced in hydrogenosomes only by substrate-level phosphorylation. Like mitochondria, hydrogenosomes (including hydrogenosomes of anaerobic fungi) are surrounded by a double-membrane (Benchimol et al., 1997), and produce ATP. In contrast to mitochondria, hydrogenosomes produce molecular hydrogen through fermentations, lack cytochromes and lack DNA.

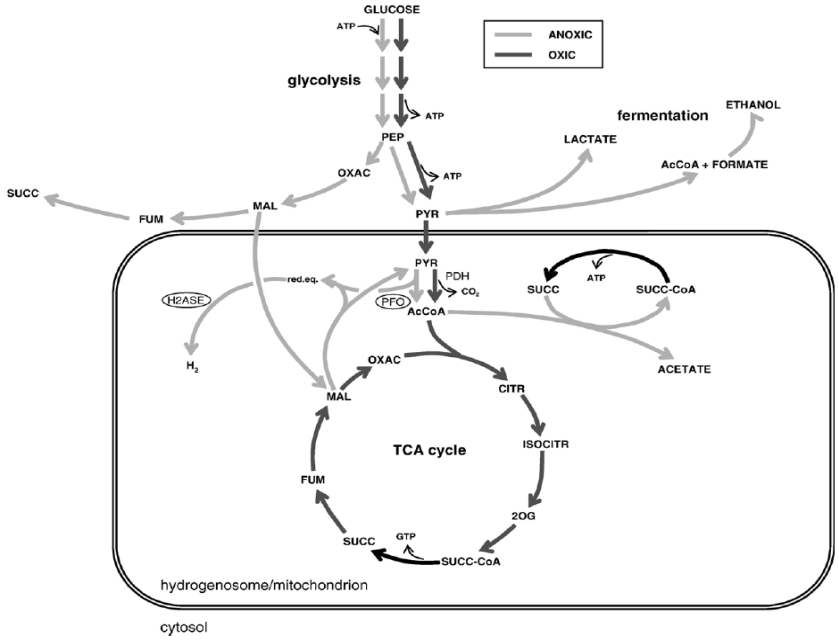

Figure 4 - Generalised pathway of carbohydrate degradation in aerobic eukaryotes compared to that of anaerobic fungi. Aerobic degradation is indicated by dark grey arrows and anaerobic degradation by light grey arrows. AcCoA, acetyl-CoA; CITR, citrate; FUM, fumarate; H2ASE, hydrogenase; ISOCITR, isocitrate; MAL, malate; 2OG, 2-oxoglutarate; OXAC, oxaloacetate; PEP, phosphoenolpyruvate; PFO, pyruvate: ferredoxin oxidoreductase; PDH, pyruvate dehydrogenase; PYR, pyruvate; red.eq., reducing equivalents; SUCC, succinate; SUCC-CoA, succinyl-CoA. Succinyl-CoA synthetase is indicated by black arrows in both routes. (Dacks, J.B. 2006).

Hydrogenosomes followed probably an alternative way to adapt to anaerobic conditions. The available functional and phylogenetic evidences offer two different views on the evolutionary history of these organelles. One hypothesis (Yarlett and Hackstein, 2005) suggests that hydrogenosomes of anaerobic fungi, trichomonads and ciliates are

substantially different and evolved independently from each other. The other hypothesis (Benchimol, 2009) demonstrates a similarity between hydrogenosomes of anaerobic fungi and trichomonad protozoa, considers hydrogenosomes as homologous organelles in unrelated species. Now, hydrogenosomes are generally accepted as organelles derived from mitochondria.

2.4.5 Flagella

Neocallimastigomycota (anaerobic fungi) is one of tree fungal phylum with asexual reproduction by flagellated zoospores (uniflagellated zoospores are also produced by Chytridiomycota and Blastocladiomycota). A flagellum, an organelle of locomotion, is a long slender projection from cell body with function to propel organism by beating with a whip-like motion (Cosson, 1996). Flagellated fungi are assumed to form early-diverging clades within the Fungi Kingdom, because the simple aquatic forms with flagellated spores are believed to be the fungal ancestor (James et al., 2006). The loss of flagellum was certainly an important evolutionary event leading to the diversification of terrestrial fungi. Gut fungi include uniflagellate and polylagellate taxa. In the uniflagellate species, the zoospores are predominantly uniflagellate, but sometime, it is possible that they have two or four flagella. (Fig. 5).

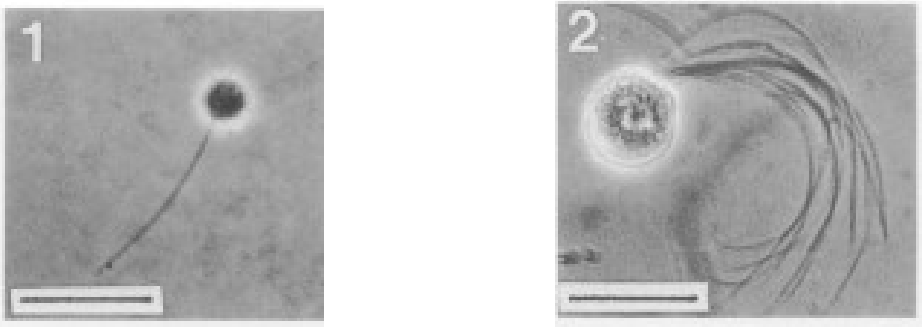

Figure 5 - 1. A uniflagellatte zoospore (*P. minutus*). **2.** A polyflagellate zoospore (*O. joyonii*). Bar, 20 μm. (Ho et al. 1995).

Zoospores of polyflagellate species always have more than four flagella. *Orpinomyces* and *Neocallimastix* sp. are polyflagellate producing zoospores with 7 – 20 flagella inserted in two rows. The zoospores of *Anaeromyces*, *Piromyces, Caecomyces* and *Cyllamyces* are uniflagellate (sometimes biflagellated) (Mountfort and Orpin 1994).

2.4.6 DNA composition

The GC content of the anaerobic fungal genome is approximately 15-20 mol% and is amongst the lowest reported in any organism (Brownlee 1994, Nicholson et al., 2005). This extreme nucleotide bias is reflected in both the coding and non-coding regions of the genome, with codon usage tending towards more AT-rich codons (Garcia-Vallvé et al. 2000). The non-coding regions are even more AT-rich with many sections expected to be near or above 95 mol% of AT content (Nicholson et al., 2005). The codon bias resulting from the low G+C content leads to a depletion in amino acids coded by GC rich codons (e.g. arginine, proline, glycine) and an overabundance of amino acids coded by AT rich codons (e.g. lysine, phenylalanine, tyrosine).

2.4.7 Enzymes

Anaerobic fungi produce the whole set of enzymes necessary for plant cell-wall degradation including cellulases, xylanases, mannases, esterases, glucosidases and glucanases. These hydrolases enable fungi to penetrate plant cell walls, access fermentable substrates not available to surface-acting bacteria, colonize the sturdy plant structures, weaken and degrade plant tissues and reduce the plant particles size. Due to the capability to colonize the recalcitrant plant components, like sclerenchym and vascular system, gut fungi are known to decompose lignin-containing plant walls, even if they do not utilize phenolics or lignin.

To decompose the complex structure of cellulose, the most abundant polysaccharide, three different classes of enzymes are necessary: endoglucanase (cellulase), spliting β-

1,4-glycosidic bonds randomly within the cellulose chain, exoglucanases (cellobiosidase or cellobiohydrolases), cleaving cellobiose from the ends of the cellulose chain and β-glucosidases (cellobiases), which convert cellobiose and other low molecular mass cellodextrins into glucose.

The xylan, the principal type of hemicellulose and second most abundant natural polysaccharide, is a polymer of β-1,4-linked xylopyranosyl units. Its conversion requires the participation of several enzymes, which are effectively secreted by anaerobic fungi. The main hydrolase is the xylanase (endoxylanase) cleaving β-1,4- xylosidic linkage randomly within the main xylan chain and xylosidase, which removes the successive D-xylose residues from the non-reducing ends. The ester bonds on xylan and the side chains are hydrolyzed by feruloyl acid esterases, acetylxylan esterases, arabinases, or β-glucuronidases depending on the type of branching group.

All genera of anaerobic fungi degrade cellulose and hemicellulose, but the range of their hydrolysation depends on the degrees of crystallinity. Moreover gut fungi exhibit genus-dependent differences in their activity towards (hemi)celluloses and recalcitrant substrates (like weat straw). *Neocallimastix* and *Piromyces* spp. are supposed to be the most active genera (Thareja et al., 2006, Tripathi et al. 2007, Dayananda et al. 2007), while *Caecomyces* sp. the least effective genus (Nielsen et al., 2002, Joblin et al., 2002).

2.4.8 Cellulosomes

Some of the hydrolytic enzymes produced by gut fungi act individually and are free in solution, whereas others are constituents of large (hemi)cellulase multienzyme complexes called cellulosomes. The Neocallimastigomycota is the only known member of the kingdom Fungi possessing cellulosomes and this can explain their cellulolytic superiority over aerobic cellulolytic fungi. These exocellular enzyme complexes are extremely active and can degrade both amorphous and crystalline cellulose (Bayer et al., 2004).

The cellulosome is characterized by the presence of two general components. The first, the nonenzymatic scaffolding protein(s) with enzyme binding site called cohesions, and

second, a variety of cellulosomal enzymes with dockerins, which interact with the cohesins in the scaffolding protein, thus forming cellulosomal complexes (Doi, 2008, Fontes and Gilbert, 2010).The enzymes organized in cellulosomal complexes containing endo- and exo-cellulases, hemicellulases and esterases have been up to now described for *Piromyces*, *Orpinomyces* and *Neocallimastix* species and, molecular biological studies, indicate that the enzymes associated with these fungal cellulosomes are modular like those of anaerobic bacteria.

2.4.9. Anaerobic fungi of the horse digestive tract

There are only few reports about the anaerobic fungi of the equine intestinal tract, but any study has analysed the distribution of this microorganism in the different parts of the hindgut. Orpin (1981), for the first time, detected anaerobic fungi in cecal samples from horse. Among five isolates of uniflagellate gut fungi, one was described as *Piromonas communis* (now *Piromyces communis*), another one as *Sphaeromonas communis* (now *Caecomyces communis*) and three morphologically different isolates were not classified. A fungus with bulbous rhizoids isolated from the feces of the horse by Gold et al. (1988) was described as new species named *Caecomyces equi*. The description of this strain is based mostly on ultrastructure details. *Piromyces mae* sp. nov. was isolated from the horse hindgut by Li et al. (1990) and the description is also based on morphological details of cell organelles, mainly kinetosomes an perikinetosomal apparatus. Another new species of anaerobic fungus, named *Piromyces rhizinflata,* was isolated even from dried feces of Saharian ass (Breton et al., 1991). The *Piromyces citronii*, sp. nov. was isolated from the cecum of one pony and three donkeys by Gaillard-Martinie et al. (1995). The metabolic study of this strain showed that, the equine strains, do not produce lactate as end fermentation product and degrade cellulose more rapidly and with a greater extent than ruminal anaerobic fungi (Julliand et al.1997). All these publications describe the morphologically and metabolically strains isolated from the horse digestive tract or feces. Unfortunately not any DNA analysis is concluded, which means that no

sequence of cultivable strains of anaerobic fungi isolated from the horse hindgut is available in public database. The molecular study of Liggenstoffer et al. (2010), analysing gut fungi in the feces from 30 different herbivores, also included horse samples and brought interesting results. Known genera of anaerobic fungi were found in hindgut Equidae samples only in limited numbers: Cyllamyces 4-12%, Neocallimastix 2%, Piromyces 0.3%, Anaeromyces 0.1-0.3%, Orpinomyces and Cyllamyces 0%. On the other hand, the two novel groups of uncultured anaerobic fungi described by Liggenstoffer et al. (2010) constituted the majority of the sequences in all studied horse samples. These results indicate that the digestive tract of Equidae is occupied by completely unknown anaerobic fungi, which differ substantially from rumen fungi. Dougal et al. (2012) compared quantitatively the abundance of anaerobic fungi in the horse cecum, right dorsal colon (RDC) and feces and described an higher concentration of fungi in the RDC and feces. The quantity of gut fungi in the cecum was three times lower than in other studied parts of the horse hingut, however results were not statistically significant. These findings demonstrate the different distribution of anaerobic fungi along the horse digestive tract without any detailed knowledge about their diversity.

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CHAPTER 3

METHODS OF INVESTIGATION OF MICROBIAL POPULATION IN THE INTESTINAL TRACT

3.1 Culture methods

The anaerobic techniques to investigate microbial population of digestive tract were described in detail, for the first time, by Hungate in 1950 (Hungate, 1950). In literature it is possible to found several studies, where the Hungate method was modified in function of the experimental requirements (Joblin 1981, Lowe et al., 1987, Miller and Wolin, 1974). The isolation of gut fungi from the rumen fluid or from the feces of herbivores is based on classical microbiological approach of picking up of fungal colonies from solidified culture media inoculated by diluted rumen content or without it (Lowe et al., 1985). For the growth of the culture, it is important to have anaerobic conditions, established in glove-box, anaerobic jar, or in serum bottles closed with a butyl rubber stopper with a crimped metal seal (Miller and Wolin, 1974) or with a plate culture technique (Lowe et al., 1985). The addition of antibiotics, to suppress bacterial germination, is essential for the growth of mycelium.

A normal medium for cultivation of anaerobic microorganisms is a complex broth containing the following components: salt solution, clarified rumen fluid, nitrogen source, haemin solution, fatty acid solution, Trypticase peptone, NaHCO, resazurin solution, bidistillate water, phosphate and/or bicarbonate buffers, vitamins, chemical reducing agents and redox indicator and glucose, cellobiose, xylose or carboxymethyl cellulose as carbon source and cystein to establish redox potential (Joblin, 1981, Caldwell and Bryant, 1966, Davies et al., 1993). The soluble and insoluble carbohydrates or wheat straw are used as energy sources. At 6 h after inoculation at 39°C (optimal temperature) it is possible to see the discrete fungal thalli. They increase in size until 20 to 24 h after inoculation, when zoospores are released from the sporangia. After their release, zoospores maintain their motility for 30 to 60 min in the immediate vicinity of the ruptured sporangium but are unable to penetrate the agar. Because the zoospores could not migrate far, at 40 to 50 h after inoculation, a colony of fungal thalli is developed at each site of zoospore release. Is possible to examine the

colony with microscope and analyse their fermentation products (VFA and ethanol) with gas-chromatography method.

It is possible to store the anaerobic fungi for long time using the cryopreservation method. Yarlett et al. (1986) proved that a long storage using liquid N_2, or solid CO_2 (-80 °C) is possible, without changes of the morphological and biochemical characteristics (time of analysis 1 year). In a successive study, for the same purpose Sakurada et al. (1995) used dimethyl sulfoxide, propylene glycol or ethylene glycol as cryoprotectants. Ethylene glycol was the most effective agent. The 80% of samples equilibrated for 15 minute in medium containing ethylene glycol and cell-free rumen fluid, survived after 1 year of storage in dry ice and at -84^{o} C.

3.1.1 Quantification

The determination of the extent of fungal participation on digestion has always met the problem of assesment of the anaerobic fungi biomass (number). The population sizes of anaerobic fungi estimated by roll tube methods using antibacterial agents (Joblin, 1981) are inexact of depending on the colony-forming ability of zoospores and not reflecting interaction between bacteria and fungi. The **direct counts** of fungal colonies on plant fragments (Windham and Akin, 1984) or agar strips (Ushida et al. 1989) incubated in the rumen, seem to be more suitable method for the estimation of the relative size of the fungal population.

Since **chitin** is present in the cell wall of gut fungi (Orpin, 1977) , this polysaccharide is used as a valid indicator of the fungal biomass for a mixed population of anaerobic fungi of *in vitro* cultures, however this method has several disadvantages casting doubts on its reliability. The accuracy of the chitin assay is affected by the conditions for the hydrolysis procedure. The percentage of the hydrolysate recovery depends upon the acid concentration, the duration of hydrolysis treatment and its temperature. Distorsion is also caused by the presence of chitin found in bacterial and protozoal fractions (Gay 1991, Rezaeian et al. 2004). Theodorou et al. (1990) developed **most probable numbers** (MPN) procedure for the enumeration of anaerobic fungi as thallus forming units

(TFUs). This technique, which relies on MPN statistical tables to provide a viable cell count, involves squeezing rumen contents through muslin and preparing a serial dilution of the filtrate in an anaerobic, antibiotic-containing, basal medium. This procedure can be used with the digesta and fecal samples and, the values obtained, are generally equal to or higher than those recorded using either of the two zoospore-counting procedures described earlier (Thedorou et al. 2005).

Nevertheless Griffith et al. (2009) employed the most probable number method for the enumeration of morphologically different groups of anaerobic fungi from the rumen digesta, and fresh or frozen-thawed feces of cattle. Some authors used enrichment cultures combined with a range of carbon sources and revealed large variation in the relative abundance of different anaerobic fungi morphotypes dependent on energy source with the highest MPN counts (> 10^6 thallus forming units per g of dry matter) on wheat straw.

3.2 Molecular technique

Problems with microbial culture methods (problematic growth and identification, as well as limitation in throughput and the long time necessary to have results) are totally or partially overcomes by using molecular methods (non-culture-dependent methods). Two important techniques have stimulated the use of the molecular disciplines as approach to the anaerobic studies: the PCR (Polymerase-Chain-reaction) and the selection of universal oligonucleotides primers specific to fungi. In the table 1 are reported the name, sequence, specificity, localization, technique and reference of the primers for the amplification of anaerobic fungi rDNA – ITS1 sequence.

Molecular techniques can be used for phylogenetic and taxonomic studies or for diagnostic applications. As said previously, the eukaryotic gene for the ribosomal synthesis (rDNA) codify four important structural components of rRNA: 28S, 5.8S, 5S

and 18S, present in all eukaryotic ribosomals. Since it has an essential function in the cell, it has a structure highly conserved and serves as reference point for evolutionary divergence studies. Another important reason for the use of rDNA as reference point is its multiple-copy repeated in tandem (Guarro et al., 1999).

Every structural unit has an external transcribed spacer (ETS) on the 18S 5' extremity and 2 internal transcribed spacers (ITS1-ITS2). All spacers are spliced out of the transcript. Between each unit and before the 5S gene there is a non-transcribed spacer (NTS) (Fig.1). The 18S, 5.8S and 28S sequences are transcribed by DNA-polymerase I, whereas the 5S gene is transcribed, in the opposite direction, by DNA-polymerase III.

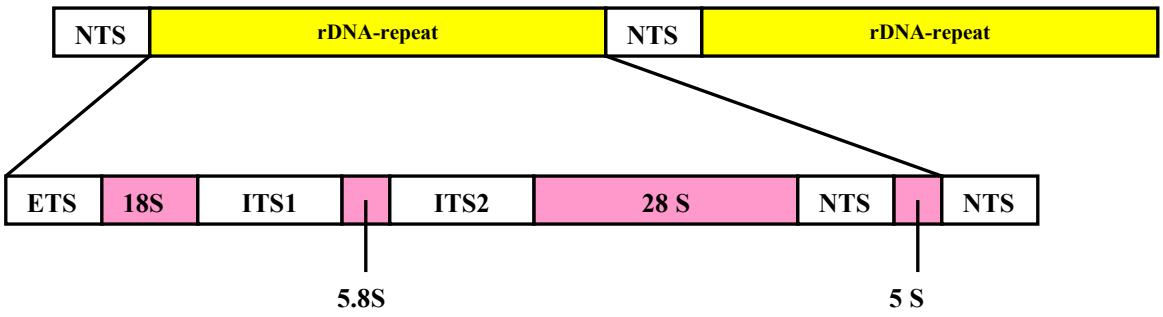

Figure 1 - rDNA gene.

The small ribosomal subunit (18S or SSU) is highly conserved in different taxa, more than 97% identical amoung genera of anaerobic fungi (Brookman et al., 2000) and thus contains little phylogenetically useful information for the subgeneric classification. For example Bowman et al. (1992) used partial 18S rRNA sequence to successfully determine phylogenetic relationships among three major classes of fungi, *Basidiomycetes, Ascomycetes* and *Chytridiomycetes*, but unsuccessful determination of different species of anaerobic fungi, which means that 18S is not a good sequence to identify different *Neocallimastigales* species.

In contrast to the small subunit, the internal transcribed spacers regions (ITS1-ITS2) show a high level of variability and can be used for the differentiation of anaerobic fungi

genera (Brookman et al. 2000; Fliegerova et al. 2004). Furthermore, the internal localization between highly conserved sequences, 18S and 5.8S, permits to use it as the universal eukaryotic primers. For example, Li and Heath (1992) used the internal spacer 1 region (ITS1) for the study of phylogenetic relationships of the anaerobic fungi from rumen and cecum of herbivorous animals. These authors found that *Anaeromyces* is more distant from *Orpinomyces*, *Neocallimastix* and *Piromyces*. Within the ITS1 sequence, four regions of major variation (Variable Regions I–IV) were flanked by regions of largely conserved sequence. The conserved sequences were found between variable regions I and II and between variable region IV and the 5.8S gene sequence. In several studies, this conserved sequence, identify the bond-site primer-DNA (Tuckwell, et al. 2005, Brookman, et al. 2000, Nicholson et al. 2010). The polymorphism of these variable regions was analysed with the secondary-structure prediction and with an algorithm method, based on hidden Markov model (HMM) analysis. Tuckewell et al. (2005) showed that, despite each variable region have a secondary structure, characterised of several different motifs (stem and loop), they are broadly conserved for each group. The 5.8S rDNA is too small and has the least variability (Brookman et al. 2000) and is not proper for phylogenetic studies.

3.2.1 PCR (Polymerase-Chain-Reaction)

The PCR is a rapid technique to identify and analyse DNA. The most important ingredients for a good amplification are the *Taq Polymerase Enzyme*, able to synthesize a new DNA filament complementary at one single DNA filament, and good primers (about 20 bp) necessary to start the amplification of the specific DNA segment from total DNA (Wilson and Walker, 2005). Other important characteristics are that only small amount of DNA are requiered, about 0.1 to 10 ng per amplification, both strands of the gene can be sequenced and the method is compatible with automated DNA sequencing instruments that utilize fluorescently labeled sequencing primers or dideoxynucleotide triphosphates (White et al., 1990). The method is based on the application of thermal cycles using the thermocycler. The cycles (about 30 total cycles)

are divided into 3 steps at different temperatures: 1. the heating process (~ 90°C) for DNA denaturation, 2. the annealing between primer and DNA target (~60°C) and 3. the elongantion (~ 72°C) of the new DNA by Taq polymerase enzyme (Fig. 2).

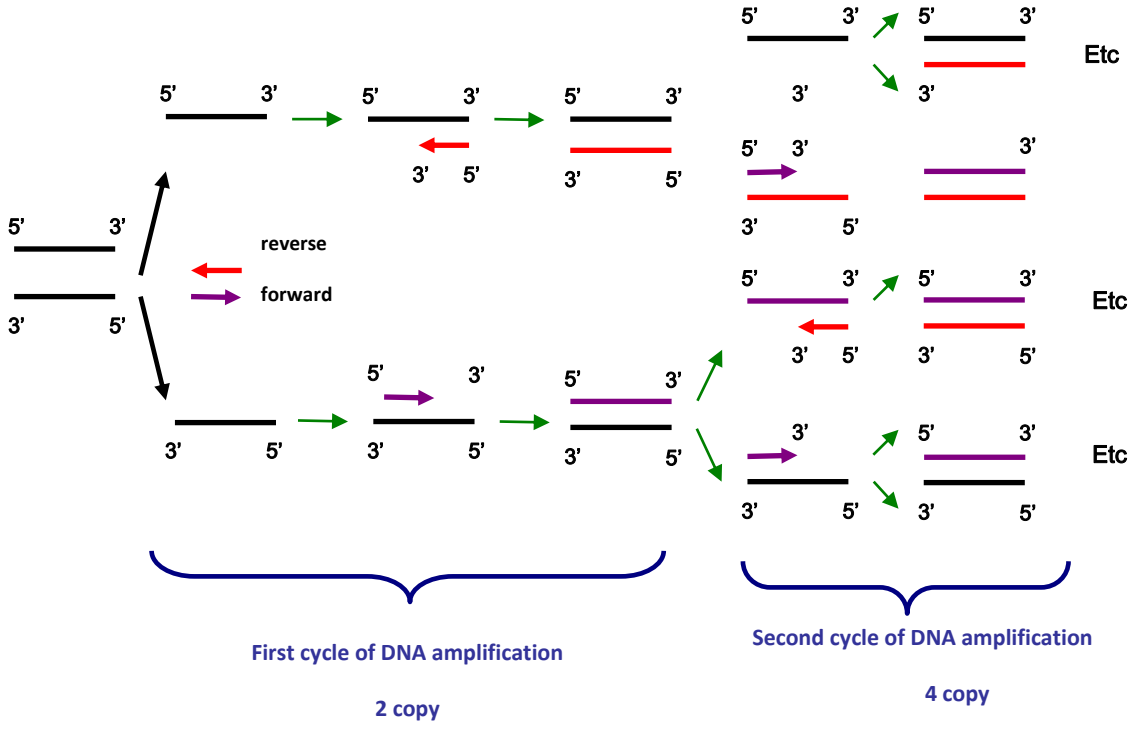

Figure 2 - Polymerase-Chain-Reaction.

3.2.2 Cloning Library

The construction of Cloning Library, is an important step necessary to assort the products obtained by the PCR with specific primers. PCR amplicons are included in the vector (plasmid or phages) by the cloning reaction and the vector is introduced into the bacterial cells creating the collection of host cells. Each of these cells contains a plasmid with a different genomic insert of DNA. The whole set of cells represents the cloning library.

As previously said, a correct design of primers is important to identify the segment of interest inside the DNA. For anaerobic fungi, several primers, targeting the internal transcribed spacer region 1, were designed (Tab.1). After the PCR, ITS amplicons are used for the fingerprinting analyses by **DGGE** (Denaturing Gradient Gel Electrophoresis), size-based selection using **Spreadex gel, ARISA** (Automated Method of Intergenic Spacer Analysis) or **RFLP** analysis (Restriction Fragment Length Polymorphism). **Q-PCR** and **Slot-blotting** are used as quantitative methods of analysis. Other techniques used for the differentiation of the microbial population are **ISH or FISH** (In situ Hybridation and Fluorescent In situ Hybridation).

3.2.3 DGGE (Denaturing Gradient Gel Electrophoresis)

DGGE is a genetic fingerprinting technique that enables the separation of double-stranded DNA fragments up to 500 bp in length, utilizing either a denaturing or temperature gradient gel. It is used to separate amplicons derived from PCR (with the same length) in function of the different GC-DNA composition and is usually employed to assess the structure of microbial communities in environmental samples without cultivation. To increase the resolution, one primer is added of a GC clamp (about 40-bp) during PCR amplification at its 5' end (Muyzer, et al. 1998), to consent to denature the amplicon, without the total separation of the fragments. Usually, DGGE is performed using a gradient 15-35% denaturant in 10% acrylamide/bis accylamide, where 100% denaturant solution containing 7 M urea and 40% (v/v) deionised formamide. The perpendicular electrophoresis is performed at 60°C and at 200 V (Brookman, et al. 2005, Nicholson, et al., 2010, Kittelmann, et al., 2012). After the electrophoresis the bands are photographed under UV transillumination. The bands of interest can be excised from the gel and re-amplified with a primer without GC-clamp of the DNA. Then the ITS1 fragments can be cloned for sequencing to enable the identification of the community members. Another possibility is to use a simple electrophoretic separation of the rDNA ITS1 fragments, using high-resolution **Spreadex gels** that enable the separation of

closely sized nucleic acid fragments. This gel is capable to resolve the fragments which differ in length by only 2–4 bp (Brookman, et al. 2005).

3.2.4 RFLP (Restriction Fragment Length Polymorphism)

RFLP is a technique, that can be performed either directly on total community DNA or on ITS1 or ITS1-4. This technique is based on using restriction enzymes capable to split the DNA in specific sites. The detection of different size bands makes information about the polymorphism of ITS regions. During this procedure the ITS region is first amplified by PCR with specific primers, precipitated with NaCl and ethanol, thus concentrated and digested with enzymes. The fragments are than separated by electrophoresis in agarose gel (Gomes et al. 2002). Fliegerova et al. (2002) analysed the polycentric rumen fungi, demonstrating that this technique can discriminate the morphologically very similar genera of anaerobic fungi.

Table 1 - Primers for amplification of anaerobic fungi rDNA - ITS1 sequence.

Name	Sequence	Specificity	Localization	Technique	Reference
MN100F MNGM2R	5'TCCTACCCTTTGTGAATTTG3' 5'CTGCGTTCTTCATCGTTGCG3'	Eukaryote Anaerobic fungi	5'end of ITS1 5'end of 5.8S	DGGE; Spreadex; Pyrosequencing ARISA	Nicholson et al. 2010 Kittelmann et al. 2012 Tuckwell et al., 2005 Denman et al. 2008
GM1F GM2R	5'TGTACACACCGCCCGTC3' 5'CTGCGTTCTTCATCGAT3'	Eukaryote Anaerobic fungi	3'end of 18S 5'end of 5.8S	*In situ* Hybridization; slot-blotting	Brookman et al., 2000 Li and Heath, 1992
ITS1F ITS400R	5'TCCGTAGGTGAACCTGCGG3' 5'ATTGTCAAAAGTTGTTTTTAAATTAT3'	Eukaryote Anaerobic fungi	5'end of ITS1	DGGE; Clones Libraries	White et al., 1990 Kittelmann et al. 2012
Neo18SF Neo5.8SR	5'6FAM-AAT CCT TCG GAT TGG CT3' 5'CGA GAA CCA AGA GAT CCA3'		3'end of 18S 5'end of 5.8S	ARISA	Edwards et al, , 2008
NeoQPCRF NeoQPCRR	5'TTG ACA ATG GAT CTC TTG GTT CTC3' 5'GTG CAA TAT GCG TTC GAA GAT T3'	Eukaryote Anaerobic fungi	partial of 5.8S	qPCR	Edwards et al., 2008
AF1482F AF100R	5'GAGGAAGTAAAAGTCGTAACAAGGTTTC3' 5'CAAATTCACAAAGGGTAGGATGATT3'	Eukaryote Anaerobic fungi	3'end of 18S 5'end of ITS1	qPCR	Denman, et al. 2006 Kittelmann et al. 2012
ITS1F ITS4B	5'CTTGGTCATTTAGAGGAAGTAA3' 5'CAGGAGACTTGTACACGGTCCAG3'	Eukaryote Fungi	3'end of 18S 5'end of 28S	Normal electroforesis; RFLP	Gardes and Bruns, 1993

3.2.5 ARISA (Automated Ribosomal Intergenic Spacer Analysis)

ARISA is a method of microbial community analysis, which provides an estimation of microbial diversity. The method involves PCR amplification of the intergenic region between the small and large subunit rRNA gene, with oligonucleotide primers targeting the conserved regions in the 18S and 5.8S genes. One of these primers is a fluorescence-tagged oligoncleotide primer. The electrophoretic step is subsequently performed with an automated system, which provides laser detection of fluorescent DNA fragments. The discrimination of these larger size fragments represents a new application for the capillary electrophoresis system employed (Fisher and Triplett, 1999). Edwards et al. (2008) as well as Denman et al. (2008) used automated ribosomal intergenic spacer analysis (ARISA) to analyse the anaerobic fungi population. Both methods were based on the amplification of ITS1 region, but each working group used different primers (see table 1). Denman et al. (2008) applied the ARISA technique for the illustration of the effect of the diet on fungal composition. ITS1-targeted ARISA method indicate the diet-depending shifts in fungal populations with greater fungal diversity in cattle fed by diets high in plant fiber (roughage) compared with diets high in soluble carbohydrates. However, the authors were able to monitor only three genera (*Neocallimastix, Piromyces*, and *Orpinomyces*). Edwards et al. (2008) used this technique to determine the dynamics of inizial colonization of forage by anaerobic fungi. These methodologies opened the way for the identification of the fungal diversity and numbers of novel sequence groups within the microbial ecosystems.

3.2.6 ISH (In Situ Hybridization)

ISH is an important molecular technique used for cytogenetic studies based on the detection of specific nucleic acid sequences in cellular structures, to study their intracellular localization, expression and adjustment. The ISH is based on the formation of stable base pairs (hybrid) between the designed nucleotide probe and the target nucleotide sequence. The ISH requires the uses of radioisotopic and non-radioisotopic

probes. It is possible to observe the correct hybridization of target DNA and probe by microscopical technique. The FISH (Fluorescent In situ hybridization) is the same technique but the labelled oligonucleotide probe is fluorescent. Brookman et al. (2000) used this technique as method for the differentiation of anaerobic fungi in rumen samples. The zoospores from *Neocallimastix* and *Piromyces* cultures maintained *in vitro* were probed using a *Neocallimastix*-specific fluorescent labelled oligonucleotide (5'-CTCGATTGAGAGTGATT-3', to bases 162-178 of the published *Neocallimastix* ITS1 sequence). A little intensity difference of the signal was observed to discriminate different samples, but the same author suggests that, due to the technical difficulties, it isn't a good method for gut fungi analysis.

3.3 Quantification

Enumerate anaerobic filamentous fungal populations is more difficult than bacteria, mainly as a result of their dual life stages: a motile free-swimming zoospore and a fibre-attached mature thallus (Joblin, 1981). The quantitative technique previously described, as the chitin content, TFUs and MPN are now totally or partially substituted with the development of enumeration molecular methods.

3.3.1 Q-PCR (Real-time PCR)

Q-PCR is a powerful tool, that allows the rapid quantification of a target DNA sequence through the design of specific primers using the same principle of a normal PCR (Freeman et al. 1999). The anaerobic fungal primer set was designed for the first time by Denman and McSweeney (2006) using multiple alignement of fungal 18S ribosomal and ITS1 gene sequences data of Brookman et al. (2000). The Neocallimastigales specific-amplicons (~ 120bp) were subsequently employed for real-time PCR assay to detect the

fungal population within cattle rumen. They found that the number of anaerobic fungi was increased after the feeding.

Relatively simple, quick and according to the authors a reliable method for the relative quantification of rumen anaerobic fungi has been published recently by Sekhatavi et al. (2009), who described *quantitative competitive PCR* **(QC-PCR)** technique based on co-amplification of the sequence of interest with a serially diluted synthetic DNA fragment of known concentration. The ITS1 fragments of anaerobic fungi amplified by previously designed primers (Denman and Sweeney 2006) were compared with standard control generated by the amplification of enterobacteria phage lambda DNA with hybrid primers containing anaerobic fungal primer sequences at the ends. A comparative quantification of these PCR products was done densitometrically as a reflection of differences in fluorescence of ethidium bromide-stained PCR fragments resolved by agarose gel electrophoresis.

3.3.2 Slot-Blotting Hybridization

This method involves the hybridization of the oligonucleotide probe to DNA samples immobilized on a nylon membrane and subsequent binding of the streptavidin-horseradish peroxidase conjugate to the captured biotin molecules. With the addition of the chemiluminescent detection reagents, the hydrogen peroxidase bounds indirectly to the DNA samples. This reaction is coupled to the oxidation of luminol, and the photons emitted are detected using autoradiography film. The size and density produced on the film are related to the amount of DNA immobilized on the membrane in each position. The comparison with the dilution series of standard DNA samples gives the assessment of the DNA quantity in the samples (Andersen, 1998). This membrane-based approach for the differentiation of *Neocallimastix and Piromyces*, using PCR-amplied ITS1 fragments, was used by Brookam et al. (2000). They showed that, by a specific Neocallimastix probe (*N. hurleyensis*), the hybridation to the *Neocallimastix* DNA is 20 times more strong than to the *Piromyces* DNA.

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Objective

The microbiology of the intestinal tract is essential for the health and welfare of the horse as well as for the prevention and treatment of intestinal diseases. The present thesis aims to provide information on symbiotic anaerobic fungal community within the equine gut by methods of molecular biology. The main goal of the comparative phylogenetic analysis of Neocallimastigomycota population of horse hindgut is to describe and compare the diversity of anaerobic fungi in the six different parts of equine digestive tract and consequently understand the physiological, symbiotic role of these Eukaryotic microorganisms in digestion processes. The general target of this work is to improve the limited knowledge about microbiology of equine hindgut system, to elucidate the role of the different gastrointestinal segments in feed fermentation and better understand the host microflora relationship within the horse.

CHAPTER 4

MATERIALS AND METHODS

4.1 Animal and diet

The samples for this study were taken from one adult 24 years old Anglo-Arabian castrated male horse reared in Sardinia (Italy). The grazing horse was fed with meadow hay and concentrate (3:1 ratio of dry matter) twice daily. The concentrate represented complementary feed with the following composition: corn, dry pellet of alfa alfa, peas, corn ground, wheat bran, cocoa peel, sunflower seed, corn flakes, barley flakes, pea flakes, soya, molasses of sugar cane, calcium carbonate, sodium chloride, sodium bicarbonate. The analytical composition is reported in the table 1. The horse was clinically healthy and didn't show any disease or intestinal disturbances within the previous 6 months.

Table 1 - Composition complementary feed.

Analytic Components %		**Additives per Kg:**		**Oligo-elements:**	**Added per Kg**
Moisture	12,50%	Oxide of Magnesium	60 mg	Iron	15,2 mg
Crude proteins	14,50%	Vitamin A E 672	4.800 UI	Iodine	0,40 mg
Crude fibre	10,80%	Vitamin D3 E 617	480 UI	Molybdenum	0,040 mg
Ether extract	3,00%	Vitamin B1	0,40 mg	Manganese	12,0 mg
Ash	5,90%	Vitamin B2	0,060 mg	Selenium	0,060 mg
Sodium	0,30%	Vitamin B6	0,24 mg	Zinc	14,0 mg
Calcium	0,65%	Vitamin B12	0,002 mg	Cobalt	0,080 mg
Magnesium	0,20%	Vitamin E	4,8 mg		
Phosphor	0,40%	Vitamin PP	4,8 mg		
		Acid D-Pantotenic	0.48 mg		

4.2 Sampling of the gut content

The samples were taken of the luminal gut contents from three different region of the large intestine: cecum, colon and rectum. In particular, from the colon we have taken the sampling from right ventral colon (RVC), left ventral colon (LVC), left dorsal colon (LDC) and right dorsal colon (RDC). The samples were collected immediately after the euthanasia. For each region of the gastrointestinal tract the content was put into the clean bag and transported on ice in the laboratory, where all the samples were freeze-dried. Samples were weighed before and after the lyophylization, to determine the percentage of moisture and dry matter contents.

4.3 DNA extraction

The freeze-dried samples of the gut content were rapidly frozen with liquid nitrogen and ground into a fine powder with a mortal and pestle. 1 g of the sample powder was resuspended in 7 ml of the following extraction buffer: 100mM Tris-HCl (pH 8.0), 20mM EDTA, 1.4M NaCl, and 2% CTAB (hexadecyltrimethylammoniumbromide). The mixture was incubated at 65°C for 1 hour with occasional inverting. After the incubation, an equal volume of chloroform (7 ml) was added to the extraction buffer with sample and mixed gently by inversion for 15 minutes. This mixture was centrifuged at 11.000 rpm for 20 minute (Thermo-Scientific SL-16R) to separate the water phase containing DNA and the chloroform phase containing the precipitated proteins. The aqueous supernatant was collected by cautious pipetting and in new tube mixed with 2,5 volumes of cold ethanol and 0.1 volume of 3M sodium acetate to precipitate the DNA. The tube was mixed gently by inversion for several times and stored at -20°C overnight (Chen et al. 2006). In this step the white suspension of DNA was already visible. The precipitated DNA was collected by the centrifugation for 20 minute at 11.000 rpm. The supernatant was discarded and the pellet was rinsed by 3 ml of 70% ethanol. This

alcohol was removed and the pellet was allowed to dry at room temperature for 10 minute. The traces of ethanol were evaporated by short freeze-drying (1 minute), because it is important to remove the ethanol solution. The DNA pellet was dissolved in 1 ml of TE buffer (10mM TRIS-HCl pH 7.4, 1mM EDTA pH 8.0) and subjected to further purification using glass milk.

Each 200µl of sample was mixed with 1 ml of the *bind buffer* (saturated solution NaCl, 10 mM Bis-Tris, pH 6.0) and 10 µl of glass milk. After the gently mixing for 5 minute, the sample was centrifuged for 5 sec at 11.000 rpm and the supernatant was removed. The pellet was carefully resuspended in 500 µl of *neet wash* (100mM NaCl, 1mM EDTA, 10 mM TRIS-HCl and 50%CH_3CH_2OH, pH 7.5). The sample was centrifugated for 5 sec at 11.000 rpm, the supernatant was removed, and the *neet wash* step was repeated for the second time. After centrifugation (5 sec at 11.000 rpm) the supernatant was removed a pipette and short freeze-drying (1 minute). Glass milk pellet with bound DNA was dissolved in 50 µl of TE buffer to release the DNA into solution. The sample was centrifuged (5 sec at 11.000 rpm) to sediment the glass milk and the supernatant containing the cleaned DNA was transferred in a new eppendorf tube. The solutions of genomic DNA from the different parts of horse digestive tract were stored at -20^{o}C before using.

4.4 PCR amplification of internal transcribed spacer region-1 (ITS1)

The cleaned genomic DNAs (diluted 10 times by destilled water) from the horse GIT were used for PCR amplification of the fungal ITS1 fragment of rDNA using the specific primers pair ITS1F (Gardes and Bruns, 1993) and NeoQPCRrev (Edwards, et al. 2008) (as reported in the table 2). The PCR reaction solution (2 µl of genomic DNA, 1µl of forward primer ITS1F, 1µl of reverse primer NeoQPCR rev, 6µl of the PCR water, 10µl of PCR mixture PPP Master Mix, TopBio, Czech Republic) was subjected to the

PCR reaction on thermocycler (GeneAmpPCR System 9700 – Applied System). The thermal cycling conditions are listed in the table 3.

Table 2 - Primer used for PCR amplification of ITS1 region

Target species	References	Sequence
Anaerobic Fungi	Neo QPCRrev Edwards et al. (2008)	5'- GTGCAATATGCGTTCGAAGATT-3'
Fungi	ITS1F Gardes and Bruns (1993)	5'- CTTGGTCATTTAGAGGAAGTAA-3'

Table 3 - PCR reaction conditions

Step	Time	Temperature	Cycles
Initial Denaturation	4 min	94°C	1
Denaturation	1 min	94°C	33
Annealing	30 sec	58°C	33
Elongation	45 sec	72°C	33
Final Elongation	2 min	72°C	1

After the PCR, the amplified products were controlled electrophoretically in agarose gel (0.8%) containing 5 µl of Ethidium Bromide. The electrophoresis was carried out in 1x TBE (40 mM Tris, 20 mM boric acid, 1 mM EDTA) buffer for 45 minutes at 110 V. The bands of the gel stained by ethidium bromide were visualized by UV light on Vilber Lourmat system (France). Two DNA bands were visible on agarose gel. The bigger band of approximate length 350 bp was cut out from the agarose gel with a clean sharp scalpel and subjected to the purification process using the **QIAquick Gel Extraction Kit** (Qiagen, Germany). The piece of cut gel was inserted in a clean tube and weighted. 3 volumes (of the weight of the gel) of Buffer QC were mixed with 1 volume of gel and

the mixture was incubated at 50°C for 10 minutes. The tube was inverted every 2-3 minutes to help to dissolve the gel. Then, 1 volume of isopropanol was added, gently mixed and incubated for 5 minute at room temperature. The solution was distributed into the QIAquick spin column, centrifuged for 1 minute (10 000 rpm) and the flow-through was discarded. The spin column was then washed by 750 µl of Buffer PE, centrifuged for 1 minute (10 000 rpm) and the flow-through was again discarded. To remove the residual solution containing ethanol, the empty column was centrifuged for another minute (10 000 rpm). The column was transfered into a clean microtube and the DNA (fragment ITS1) was washed out using 100 µl of Buffer EB (10 mM Tris-Cl, pH 8.5). This DNA solution was mixed with 250 µl of ethanol and 10 µl of 4M LiCl and stored overnight at -20^{o}C. The precipitated ITS1 fragment was centrifuged for 30 minutes at 12 000 rpm at 4^{o}C. The pellet was dried at room temperature and dissolved in 10 µl of PCR clean water. 1.5 µl of this concentrated ITS1 fragment was checked using agarose gel electrophoresis and an appropriate volume (minimum of 0.5 µl, maximum of 4 µl) was used for the preparation of a clone library.

4.5 Preparation of clone library

The **TOPO® TA Cloning® Kit for Sequencing (Sigma-Aldrich K4575-01)** was used for the preparation of the fungal ITS1 clone library. The ITS1 fragment was inserted into the plasmid vector pCR™4-TOPO; the components of the reaction mixture are listed in table 4. The reagents were mixed according to the order shown in the table 4.

Table 4 - Reagents necessary for the cloning reaction

Reagents (storage -20°C)	**Volume**
Product PCR (ITS1 fragment)	0,5 – 4 µl
Salt solution	1 µl
Water	Add to a total volume 5 µl
Plasmid vector pCR™4-TOPO	1 µl
Final Volume	6 µl

The microtube with this solution was mixed gently and incubated for 30 minute at room temperature. After the incubation, the solution of circle plasmid containing already the ITS fragment was either frozen (-20°C) or 3 µl of solution were directly used for *Escherichia coli* transformation procedure. The competent cells of *E. coli* TOP10 already supplied with kit were defrosted, mixed with 3 µl of solution with plasmid pCR™4-TOPO containing ITS1 fragment and incubated on ice for 30 minutes. The plasmid was introduced into the *E. coli* cells by a heat-shock for 30 seconds at 42°C without shaking. The tube was then immediately transferred to ice for 3 minutes. 250 µl of S.O.C medium solution (2% Tryptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM MgSO4, 20 mM glucose) were added at room temperature and the tube was shaken in horizontal position at 200 rpm at 37°C for 2 hours (maximal time). During this incubation the Luria-Bertani (LB) agar medium containing ampicillin (50µl /ml) was prepared (see table 5) and poured into Petri dishes.

After 2 hours of sample incubation, 10-50 µl of the sample were spread on the selective plates, which were incubated at 37°C overnight. When the transformation was succesfull, visible colonies appeared on Petri plates. The design of plasmid vector pCR™4-TOPO ensures that only the positive colonies of *E.coli* with the vector containing the ITS insert will grow. Each single colony was picked and inoculated into 1,2 ml of liquid LB medium with 75µg/ml of amplicillin. After overnight incubation at 37^{o}C, 0.5 ml of sterile glycerol was added into each tube containing the picked up

colony and stored at -20°C for further analysis. It is important that each eppendorf tube has specific number.

Table 5 - Recipe LB for 1 litre of bidistillate water.

Ingredients	**Volume**
Bactotryptone	10 g
Bactoyestextract	5g
NaCl	10 g
Glucose	2 g
Agar (only for Petri dishes)	3 g in to 200 ml of water

4.6 Analysis of positive clones

The analysis of positive/negative clones of *E.coli* was performed using the kit **REDExtract-N-Amp™ Tissue PCR Kit** (Sigma XNAT-1KT) according to following procedure.

300 µl of defrosted samples of transformed *E. coli* were inoculated in the falcon tube with 10 ml of LB medium with 100 µg/ml of ampicillin, and incubated at 37°C overnight. 1 ml of each *E. coli* culture was subjected to analysis for the detection of positive/negative clones. 1 ml of bacterial culture was centrifuged at 11.000 rpm for 5 minutes. The supernatant was removed and the pellet was resuspended in to 25 µl of *extraction solution* (Sigma Aldrich – E7526), incubated at room temperature for 10 minutes and then at 95°C for 3 minutes. After this quick lysis step, 25 µl of *neutralization solution* (Sigma Aldrich – N3910) was added. The tube was centrifuged for 2 minutes at 11.000 rpm. The supernatant phase represented the plasmid DNA necessary for the PCR analysis. The remaining volume in the falcon tube (9 ml of *E. coli* culture) was centrifuged at 11.000 rpm for 10 minutes. The flow-through was discarded,

the pellet was added of 1 ml of NaCl and stored at 4°C for 1 night for the further analysis.

The aim of the PCR analysis was to detect the length of insert (ITS1 fragment) inside the plasmid vector. The primers targeting the specific sequences of plasmid pCR™4-TOPO were used in PCR mixture, listed in the table 6. The thermal cycling conditions of this reaction are listed in the table 7.

Table 6 - PCR Mix for detection of ITS insert inside plasmid vector

Reagent	**Sequence**	**Volume**
Plasmid DNA		2 µl
RedExtractAMP polymerase (Sigma XNAT-1KT)		5 µl
Primer M13 Forward	5´-GTAAAACGACGGCCAG-3´	1 µl
Primer M13 Reverce	5´-CAGGAAACAGCTATGAC-3´	1 µl
Water		1µl
Total Volume		**10 µl**

Table 7 - The thermal cycling conditions for detection of ITS insert inside plasmid vector

Step	**Time**	**Temperature**	**Cycles**
Initial Denaturation	2 min	94°C	1
Denaturation	45 sec	94°C	33
Annealing	45 sec	45°C	33
Elongation	2 min	72°C	33
Final Elongation	4 min	72°C	1

After the amplification, the samples were loaded on the agarose gel (0.8%) with 5 µl of Ethidium Bromide. One little well was reserved for molecular marker *NorgenBiotek*

Corporation (catalogue number 11500) LowRanger 100bp DNA Ladder (100 – 2.000bp). The electrophoresis was carried out at 110V for 45 minutes. The bands of gel were visualized by the transilluminator (Gel Doc™ XR-System BioRad). The positive samples were represented by plasmid insert of approximate length ~300-400 bp. From all the positive *E. coli* clones, the plasmid DNA containing ITS1 insert was isolated as described in next paragraph.

4.7 Isolation of plasmid DNA with ITS1 insert from *E. coli*

From all positive *E. coli* clones, the plasmid DNA containing ITS1 insert was isolated using the **GenElute™ HP Plasmid Miniprep Kit** (NA0160-KT).

The pellet from 9 ml of grown *E. coli* culture (see above) dissolved in 1ml of 0.9% NaCl was centrifuged for 2 minute at 11.000 rpm and the supernatant was discarded. The pellet was resuspended in 200 µl of cold *resuspension solution* containing Ribonuclease A up to homogeneous solution. 200 µl of the *lyse solution* were added and mixed by gently inversion for 6-8 times. 350 µl of the *neutralization solution* were added, mixed properly and the tubes were centrifuged for 10 minute at 11.000 rpm to sediment the precipitated cells debris. The clear supernatant phase was transferred into the kit specific columns, which were treated by the *binding solution*. These columns were centrifuged for 1 minute (11.000 rpm) and the flow-through was discarded. 750 µl of *wash solution 2* were added and centrifuged for 1 minute at 11.000 rpm. The flow-through was discarded and to remove the traces of ethanol the columns were again centrifuged for another minute. The columns were transferred in clean eppendorf tubes and 100 µl of the *eluation solution* were added. After the last centrifugation (2 minutes at 11.000 rpm) the plasmid DNA was eluted from the column filter and the presence of ITS1 insert inside the plasmid DNA was checked again by the restriction analysis.

4.8 Control of ITS1 inserts by restriction analysis of plasmid DNA

The presence of ITS1 fungal fragment inside the plasmid vector was checked again before sequencing because of the risk of false positive reaction of the PCR method for discrimination of positive and negative samples (see paragraph 4.6). The PCR method is very sensitive and the contamination (from air, solutions, pipette tips) cannot be excluded. The insert inside plasmid vector is surrounded by cleavage sites (Fig. 2, chapter 5), but only the restriction with the enzyme EcoRI splits out the insert from the plasmid.

9 µl of plasmid DNA isolated as described in the paragraph 4.7 were mixed with 1 µl of the *NEBuffer EcoRI* (100 mM Tris-HCl, 50 mM NaCl, 10 mM MgCl2, 0.025% Triton® X-100, pH 7.5) and 1 µl of *Restrition enzyme EcoRI* (BioLabs Inc. R0101L) and incubated at 37°C overnight. The length of the restriction fragments was checked electrophoretically in agarose gel (110 V for 25 minute). The positive samples contained the fragment of approximate length 350 pb.

4.9 Sequence analysis of ITS1 fragments

The plasmids with ITS1 insert of proper length were subjected to sequence analysis. The DNA concentrations were measured by NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, U.S.A.) and the sample was prepared for sequencing at concentration 500 ng/µl. Inserts of approximate length ranging from 270 to 380 bp were sequenced using ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, U.S.A.) and analysed on ABI 310 PRISM and ABI 3730 Genetic Analyzers (Applied Biosystems, U.S.A.). T3 primer (ATTAACCCTCACTAAAGGGA) was used for sequencing, because this oligonucleotide has priming site on plasmid vector

pCR™4-TOPO. The plasmid vector sequences were trimmed and ITS1 sequences were adjusted if necessary using software Chromas Lite (http://technelysium.com.au/).

4.10 Sequence alignment and phylogenetic tree construction

The sequence fragments were assembled and aligned using MEGA 5.2 (Molecular Evolutionary Genetics Analysis) software program (Tamura, et al. 2011). The sequences which were not generated in this study were retrieved from the GenBank at http://www.ncbi.nlm.nih.gov/. The sequences used as taxon groups are reported in the table 8.

The phylogenetic trees have been constructed using maximum likelihood statistic method and nearest–neighbor Interchange (NNI) ML heuristic method. The bootstrap values are based on 1000 replicates. Only the sequences with high similarity (> 95%) with the ITS1 sequence available in public databases, were used for the phylogenetic tree. For each part of the horse GIT tract a separate tree was constructed. The representatives of all clades were included in the final phylogenetic analysis for the construction of the tree, describing phylogenetic affiliation of the anaerobic fungi sequences in the horse lower digestive tract.

The cluster analysis was performed with MINITAB 12.1 version and the similarity was calculated by the pearson correlation coefficient and grouping was performed by hierarchical cluster analysis using group average linkage.

Table 8 - GenBank sequences used for the phylogenetic analysis

Name	GenBank accession number
Anaeromyces sp. JB-1999	*(AF170187)*
Anaeromyces sp. FFEX4	*(FJ483846)*
Cyllamyces sp. AF-CTS-CHCy1	*(FJ501277)*
Orpinomyces sp. OUS1	*(AJ864475)*
Neocallimastix frontalis isolate NCS1	*(AF170194)*
Neocallimastix frontalis isolate MCH-3	*(AF170192)*
Neocallimastix frontalis strain SR4	*(AY429664)*
Piromyces polycephalus	*(AF492019)*
Piromyces sp. JB-1999	*(AF170203)*
Caecomyces communis	*(DQ067605)*
*Horse 04IPCIR **	*(GQ693057)*
*Horse 01APUGS **	*(GQ826454)*
*Horse 04H591J **	*(GQ692728)*
*Horse 1BLJOL **	*(GQ829534)*
Pichia kudriavzevii (as outgroup)	*(AB369918)*

**Reference Liggenstoffer et al. 2010- GenBank name: Uncultured Neocallimastigales.*

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GenEluate HP Plasmid Miniprep Kit. *Catalog Numbers NA0150S, NA0150 and NA0160* Sigma-Aldrich.

Restrition enzyme EcoRI Kit (BioLabs Inc. R0101L)

QIAquick Gel Extraction Kit. *Catalog Number 28704 and 28706.* QIAGEN

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S. MEGA5: *Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods*. Molecular Biology and Evolution (2011) Volume: 28 Issue: 10 Pages: 2731-2739.

TOPO® TA Cloning® Kit for Sequencing. *Five-minute cloning of Taq polymerase-amplified PCR products for sequencing. Catalog numbers K4530-20, K4575-J10, K4575-01, K4575-40, K4580-01, K4580-40, K4595-01, K4595-40, K4575-02, 450030.* Invitrogen by life Technologies.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 Amplification of fungal ITS1 fragment from genomic DNA of intestinal content

The ITS1 (internal transcribed spacer), is the sequence of eukaryotic rRNA connecting 18S rRNA component of the small eukaryotic ribosomal subunit with non-coding 5.8S rRNA component of the ribosomal large subunit. It is the fragment with variable sequences recommended as the proper marker for random target polymerase chain reaction (PCR) in the environmental biodiversity screening and phylogenetic studies of fungi. For our phylogenetic study of the anaerobic fungi in horse hindgut, we used the combination of fungal universal (Gardes and Bruns 1993) and Neocallimastigales specific (Edwards et al. 2008) primers (see chapter 4 - paragraph 4.3 and 4.4). These oligonucleotides were applied on genomic DNA isolated from digesta of the following six different parts of horse digestive tract: cecum, right ventral colon (RVC), left ventral colon (LVC), left dorsal colon (LDC), right dorsal colon (RDC), and rectum (feces). The ITS1 fragments (and partial 5.8S rDNA region) of approximate length about 350 bp were successively amplified from all studied samples (Fig. 1). PCR products represent the mixture of ITS1 fragments of different anaerobic fungi. For further analysis, the ITS1 fragments were concentrated, purificated and used for the preparation of clone libraries, which enabled the separation of ITS1 spacers and the consequent sequencing.

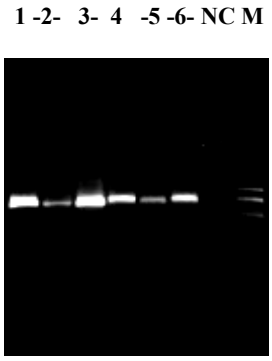

Figure 1 - Agarose gel electrophoresis of ITS1 fragments amplified from genomic DNA isolated from digesta of: lane **1**. cecum, lane **2.** right ventral colon (RVC), lane **3.**.left ventral colon (LVC), lane **4**. left dorsal colon (LDC), lane **5.** right dorsal colon (RDC), lane **6.** feces, lane **7.** negative control (NC), lane **8.** marker (**M**) NORGEN BIOTEK CORPORATION – (catalogue number 11500) LowRanger 100bp DNA Ladder (100 – 2.000bp).

5.2 Preparation of clone libraries

The ITS1 amplicons from each segment of the horse hindgut were ligated into plasmid vector pCR4 TOPO (Fig. 2) and introduced by transformation into *E. coli* strain TOP 10 to construct 6 clone libraries (paragraph 4.5). Each clone of *E. coli* thus contained the plasmid vector with individual ITS1 fungal fragment. The plasmids were isolated from *E. coli* clones and checked for the presence of ITS1 insert by PCR reaction with the primer set M13 (see paragraph 4.6). Only the plasmids with inserts of about approximate length 300-400 bp (dimension of ITS1 and partial 5.8S rDNA insert) were considered positive and were subjected to DNA sequencing (Fig. 3).

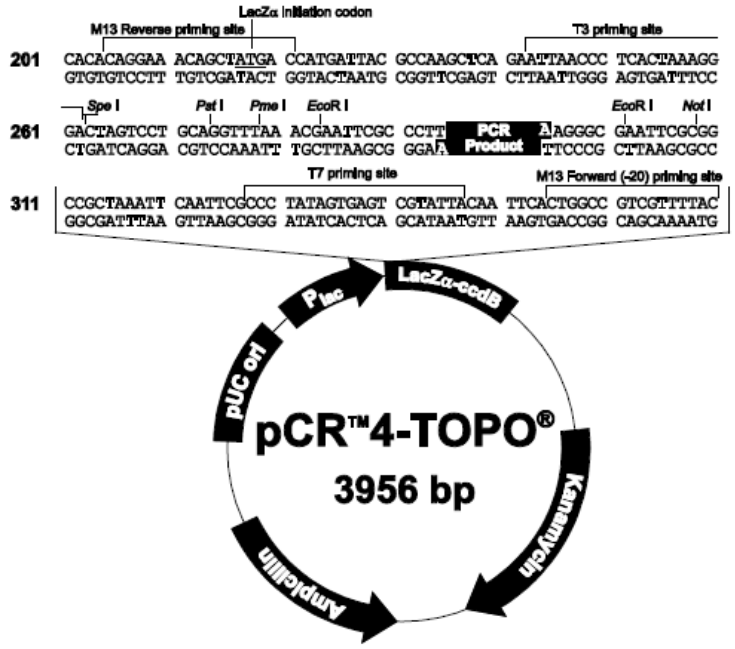

Figure 2 - Map of pCR™4-TOPO®

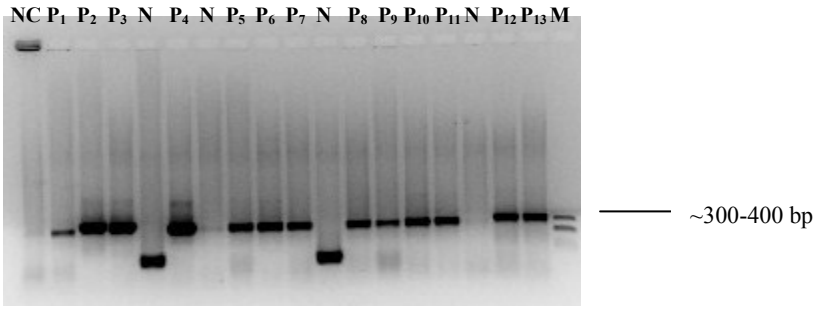

Figure 3 - Agarose gel electrophoresis (0,8%) with ethidium bromide. Fungal ITS1 and partial 5.8S sequences amplified from plasmids by primers M13. **NC**: negative control; **P_{1-13}**: positive samples; **N:** negative samples; **M**: marker lane. Bands with ~300-400 bp represent positive samples.

To eliminate the possible contamination problems and avoid the false positive samples, each plasmid DNA was checked again for the presence of ITS1 fragments by restriction analysis, using enzyme *EcoRI* capable to split off the ITS1 insert and plasmid (Fig.4).

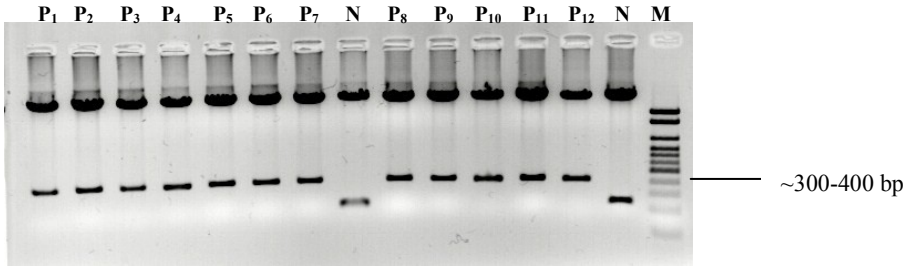

Figure 4 - Agarose gel electrophoresis (0,8%) with ethidium bromide. Fungal ITS1 and partial 5.8S sequences split off the plasmid using EcoRI enzyme. **P_{1-12}**: positive samples; **N:** negative samples (insert is too short); **M:** marker lane. Bands with ~300-400 bp represent positive samples.

All positive samples (plasmid with ITS1 insert) were sequenced. Only the sequences with high similarity (> 95%) with the ITS1 sequences available in public databases, were used for the phylogenetic analyses (paragraph 4.10).

5.3 The anaerobic fungi of the cecum content

In total, 343 clones of *E.coli* were randomly selected from the cecum ITS1 clone library and their plasmid DNA was checked for the presence of ITS1 inserts. 47% of the clones were either false positive or contained too short inserts. The other 53% of the samples bore plasmid with inserts of proper length. Their sequences were used for the phylogenetic analysis.

181 new ITS sequences (complete ITS1 and partial 5.8S rDNA) were generated from the total genomic DNA of intestinal content of horse cecum. All the sequences were compared to the GenBank nucleotide database using the BLAST search facility. Our data were aligned together with the following sequences retrieved from GenBank: *Anaeromyces sp. JB-199, Anaeromyces sp. FFEX4, Cyllamyces sp. AF-CTS-CHCy1, Orpinomyces sp. OUS1, Neocallimastix frontalis isolate NCS1, Neocallimastix frontalis isolate MCH-3, Neocallimastix frontalis strain SR4, Piromyces polycephalus, Piromyces sp. JB-1999, Caecomyces communis.* These reference sequences generated from axenic cultures represent all six known cultivable genera of anaerobic gut fungi. Moreover the four representative environmental sequences of uncultured anaerobic fungi from horse feces were included in our phylogenetic alignement. These sequences represent the new groups of anaerobic fungi discovered by Liggenstoffer et al. (2010) and described as NG 1 (Horse 04H591J, Horse 04IPCIR), NG 2 (Horse 01APUGS) and NG 3 (Horse 01BLJOL) see their GenBank accession number in chapter 4 - table 8. *Pichia kudriavzevii* was selected as outgroup taxon. 22 sequences generated in this study, representing different operational taxonomic units (OTU), were selected and included in the phylogenetic tree illustrated in figure 7.

Based on BLAST determination all 181 fungal sequences generated from the cecum digesta have been assigned to the order Neocallimastigales, however 96% of the sequences were affiliated with high identity (>98%) with uncultured Neocallimastigales, 3 of these unknown sequences exhibited lower identity (93 - 96%). Only 4% of our samples were classified on genus level. 1% of the total sequences were assigned to the

genus *Neocallimastix*, 1% to the genus *Orpinomyces*, and 2% of the sequences were highly similar to the genus *Anaeromyces*. Surprisingly no sequences (with similarity >98%) were classified as *Piromyces, Caecomyces,* or *Cyllamyces.* These data are graphically depicted in the figure 5.

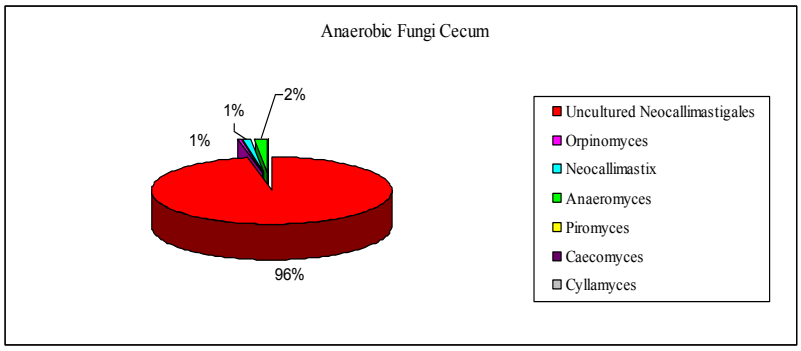

Figure 5 - Overview of the classification of all ITS1 sequences of anaerobic fungi found in the cecum in this study based on the BLAST search.

The uncultured Neocallimastigales clones generated in this study from the cecum content were compared with the new groups of uncultured Neocallimastigales described by Liggenstoffer et al. (2010) in the horse feces. As shown in the figure 6, 57% of ITS1 sequences from our cecum library (100 clones) were closely related to group NG1, but not any similarity was found with the sequences of the groups NG2 and NG3.

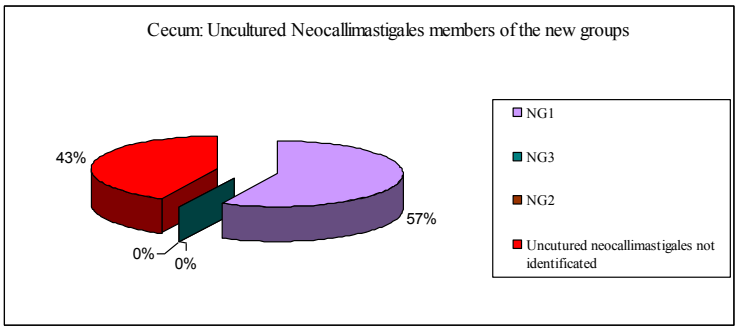

Figure 6 - Overview of similarity of cecum sequences of uncultured Neocallimastigales (96% of the total sequence encountered in the cecum in this study) with sequences of the new groups NG1, NG2 and NG3.

The remaining 43% of uncultured Neocallimastigales sequences from the cecum were phylogenetically not closely related to each other and were embedded among used reference strains of anaerobic fungi. Based on these results, the anaerobic fungi found in the cecum in this study, could be arranged in six clades. Clade **a**: sequences associated with the NG1 group of Liggenstoffer et al. (2010), clade **b**: sequences associated with genus *Neocallimastix*, clade **c**: sequences distantly associated with genus *Caecomyces*, clade **d**: sequences closely associated with genus *Orpinomyces*, clade **e**: sequences associated with genus *Piromyces*, clade **f**: phylogenetically distant sequences (Fig. 7)

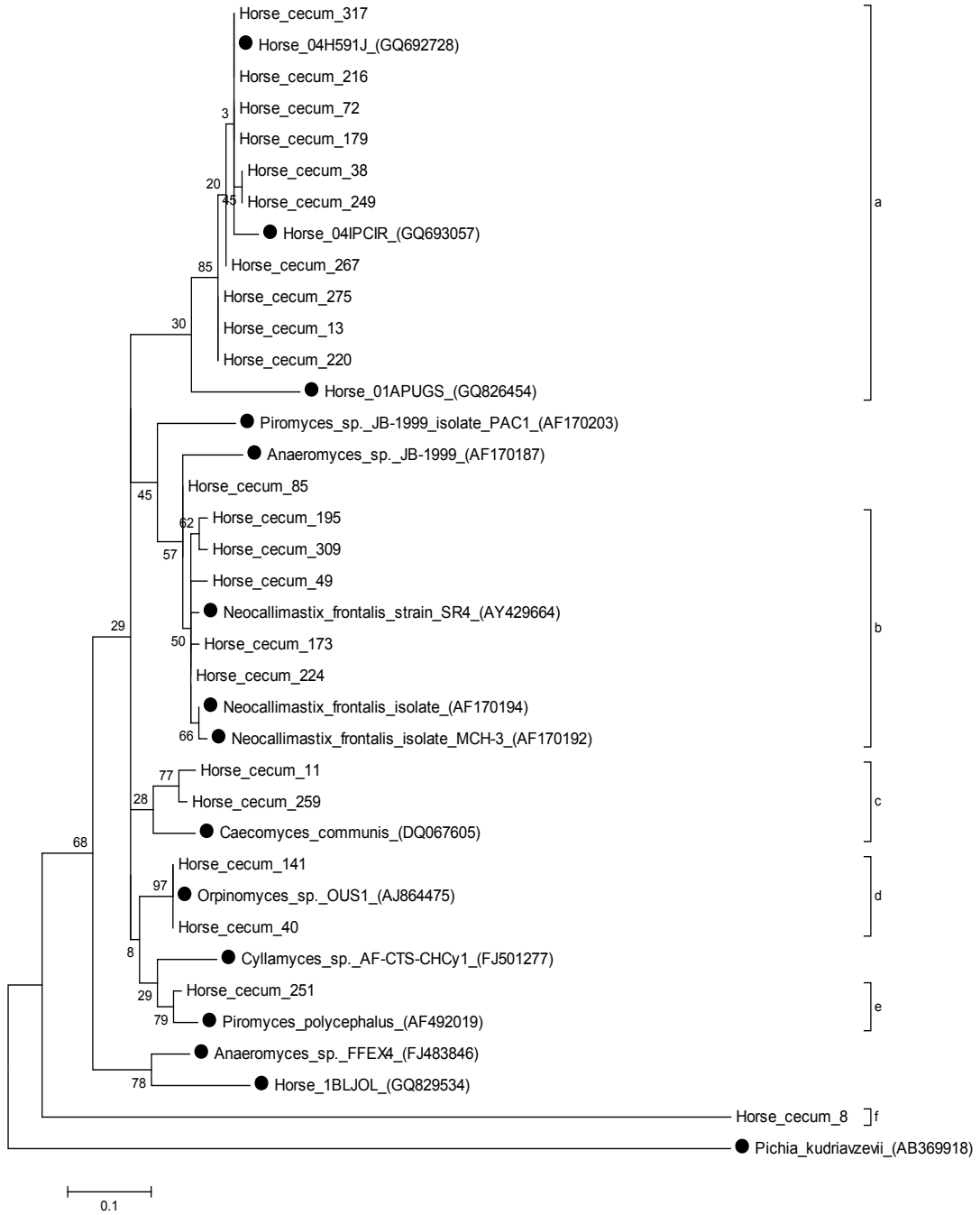

Figure 7 - Phylogenetic tree of anaerobic fungi sequences found in the cecum and analyzed in this study. Reference sequences of anaerobic fungi (with black dots) were retrieved from GenBank and phylogenetically compared with 22 sequences generated in this study and representing different OTUs. The tree was constructed using nearest–neighbor Interchange (NNI) ML heuristic method and maximum likelihood statistic method. Bootstrap values are based on 1000 replicates. **a.** NG1 cluster; **b.** Neocallimastix frontalis subgroup; **c.** Caecomyces communis subgroup; **d.** Orpinomyces sp subgroup.; **e**. Piromyces polycephalus subgroup; **f**. unknown clone. The corresponding ITS1 and partial 5.8S region of the Ascomycetous *Pichia kudriavzevii* was used as outgroup. GenBank accession numbers of reference sequences are given in parentheses.

5.4 The anaerobic fungi of the righ ventral colon (RVC) content

In total, 348 clones of *E.coli* were randomly selected from the RVC ITS1 clone library and their plasmid DNA was checked for the presence of ITS1 inserts. 50% of the clones were either false positive or contained too short inserts, the half of the samples bore plasmid with inserts of proper length. Their sequences were used for the phylogenetic analysis.

175 new ITS sequences (complete ITS1 and partial 5.8S rDNA) were generated from the total genomic DNA of intestinal content of the right ventral colon. All the sequences were compared to the GenBank nucleotide database using the BLAST search facility. The alignment was performed in the same way with the same reference strains as in the paragraph 5.3. 19 sequences generated in this study, representing different operational taxonomic units (OTU), were selected and included in the phylogenetic tree as illustrated in the figure 10.

Based on BLAST determination all 174 fungal sequences generated from the right ventral colon digesta have been affiliated with high identity (>98%) with uncultured Neocallimastigales, only 1 clone exhibited lower identity (94%). No sequences were assigned to any of the six know genera of anaerobic fungi (Fig. 8).

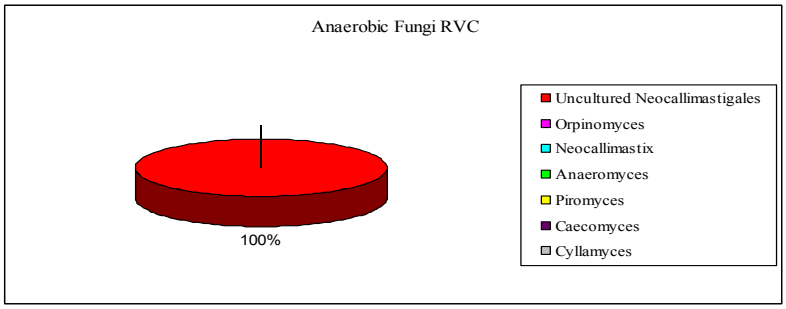

Figure 8 - Overview of the classification of all ITS1 sequences of anaerobic fungi found in the right ventral colon in this study based on the BLAST search.

The uncultured Neocallimastigales clones generated in this study from RVC content were also compared with the new groups of uncultured Neocallimastigales described by Liggenstoffer et al. (2010) in the horse feces. As shown in the figure 9, 58% of ITS1 sequences from our RVC library (102 clones) were closely related to the group NG1, 1% of our sequences were related to the group NG3 and no clone was similar to the sequences of the group NG2.

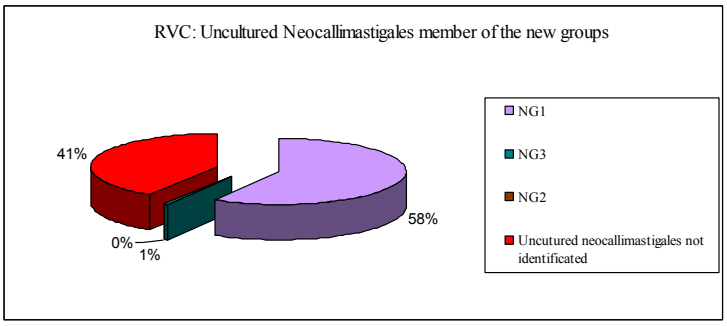

Figure 9 - Overview of similarity of RVC sequences of uncultured Neocallimastigales (100% of sequences encountered in the RVC in this study) with sequences of the new groups NG1, NG2 and NG3.

The remaining 41% of uncultured Neocallimastigales sequences from RVC were phylogenetically not closely related to any of known genus of anaerobic fungi. Based on these results, the anaerobic fungi found in the right ventral colon in this study could be arranged only into three clades. Clade **a**: sequences associated with the NG1 group of Liggenstoffer et al. (2010), clade **b**: sequences distantly related to the genus *Caecomyces*, and clade **c**: phylogenetically distant unknown sequences (Fig. 10). Compared with cecum, the anaerobic fungi of RVC exhibit lower diversity and no similarity with the cultured strains of gut fungi.

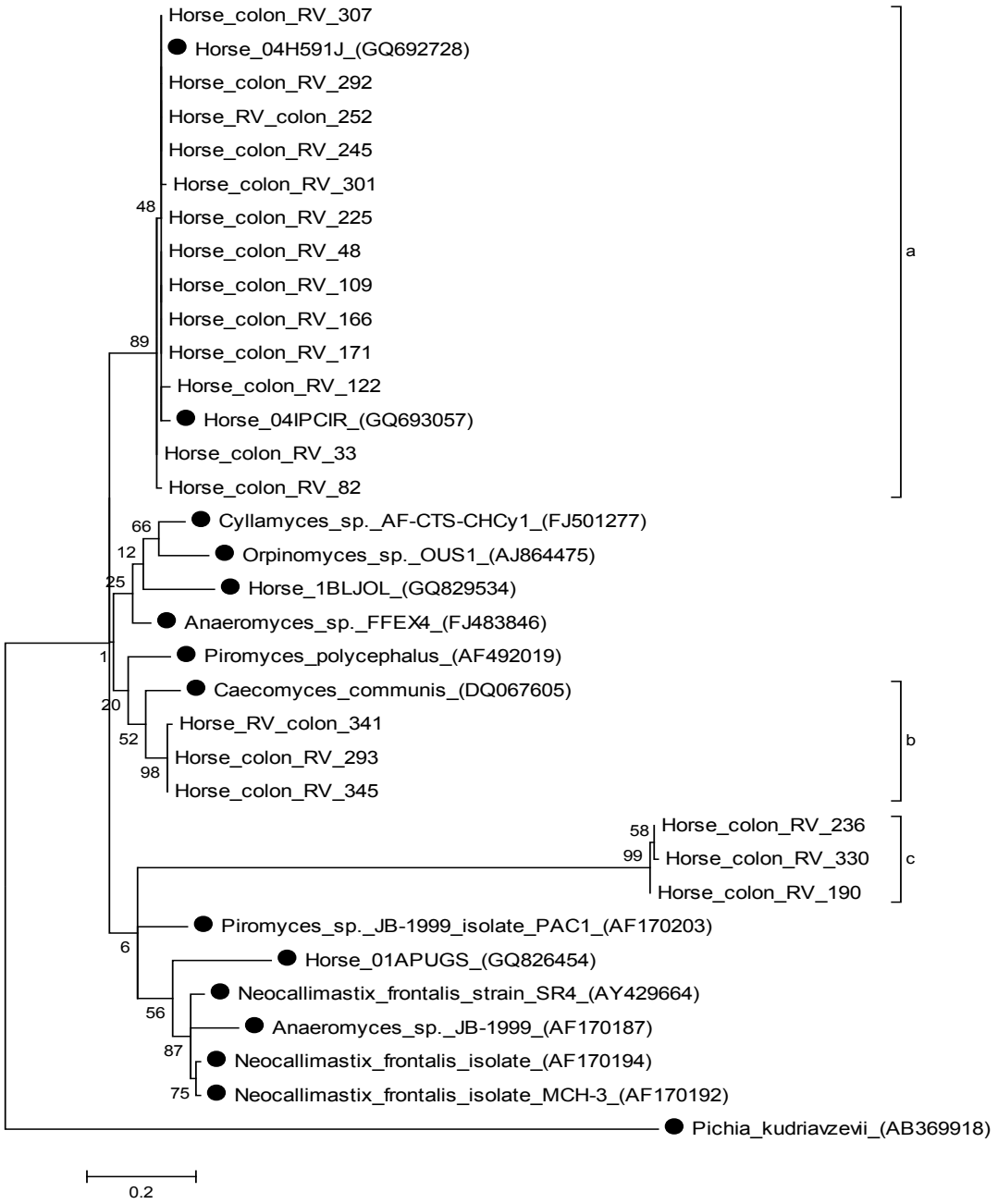

Figure 10 - Phylogenetic tree of anaerobic fungi sequences found in the right ventral colon (RVC) and analyzed in this study. Reference sequences of anaerobic fungi (with black dots) were retrieved from GenBank and phylogenetically compared with 19 sequences generated in this study and representing different OTUs. The tree was constructed using nearest–neighbor Interchange (NNI) ML heuristic method and maximum likelihood statistic method. Bootstrap values are based on 1000 replicates. **a.** NG1 cluster; **b.** group distantly related to Caecomyces communis; **c.** cluster of unknown anaerobic fungi. The corresponding ITS1 and partial 5.8S region of the Ascomycetous *Pichia kudriavzevii* was used as outgroup. GenBank accession numbers of reference sequences are given in parentheses.

5.5 The anaerobic fungi of the left ventral colon (LVC) content

In total, 326 clones of *E.coli* were randomly selected from the left ventral colon ITS1 clone library and their plasmid DNA was checked for the presence of ITS1 inserts. 64% of clones were either false positive or contained too short inserts and only 36% of samples bore plasmid with inserts of proper length. The efficiency of cloning and transformation was evidently very low.

116 new ITS sequences (complete ITS1 and partial 5.8S rDNA) were generated from the total genomic DNA of intestinal content of the left vental colon. All the sequences were compared to the GenBank nucleotide database using the BLAST search facility. The alignment was performed in the same way with the same reference strains as in paragraph 5.3. 25 sequences generated in this study, representing different operational taxonomic units (OTU), were selected and included in phylogenetic tree illustrated in the figure 13.

Based on BLAST determination all the 116 fungal sequences generated from the left ventral colon digesta have been assigned to the order Neocallimastigales, 93 clones with high identity (97-100%) and 23 clones with identity ranging from 94% up to 96%, which is still enough to be sure that ITS1 fragments belong to the anaerobic fungi, order Neocallimastigales. 51% of the sequences were affiliated with uncultured Neocallimastigales, but 49% of the sequences were classified on genus level. 22% of the total sequences were assigned to the genus *Neocallimastix*, 5% to the genus *Orpinomyces* and 22% to the uncultured *Cyllamyces* with identity 94% - 96% (no sequences had identity >98% with Cyllamyces). In the phylogenetic tree these sequences clustered distantly with *Piromyces*. No sequences were affiliated with *Piromyces, Caecomyces,* or *Anaeromyces.* These data are graphically depicted in the figure 11.

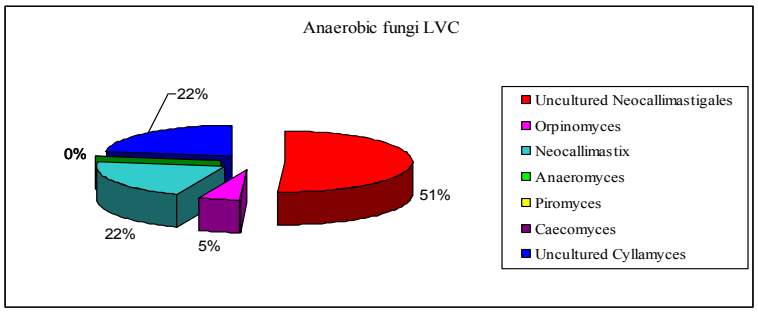

Figure 11 - Overview of the classification of all ITS1 sequences of anaerobic fungi found in the left ventral colon (LVC) in this study based on the BLAST search.

The uncultured Neocallimastigales clones generated in this study from the LVC content were also compared with the new groups of uncultured Neocallimastigales described by Liggenstoffer et al. (2010) in the horse feces. As shown in the figure 12, only 9% of ITS1 sequences from our LVC library (5 clones) were closely related to group NG1 (cluster **c** in the Figure 13), the other sequences were related neither to the group NG2 nor NG3. In the phylogenetic tree the sequences are spread among *Piromyces*, *Cyllamyces*, *Orpinomyces* and *Anaeromyces*, but the reliability of clustering is very low, so they can be considered as unknown, uncultured, anaerobic fungi.

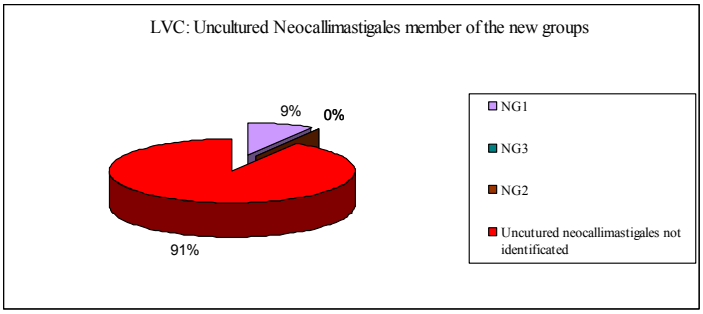

Figure 12 - Overview of similarity of LVC sequences of uncultured Neocallimastigales (51% of the total sequence encountered in the LVC in this study) with sequences of the new groups NG1, NG2 and NG3.

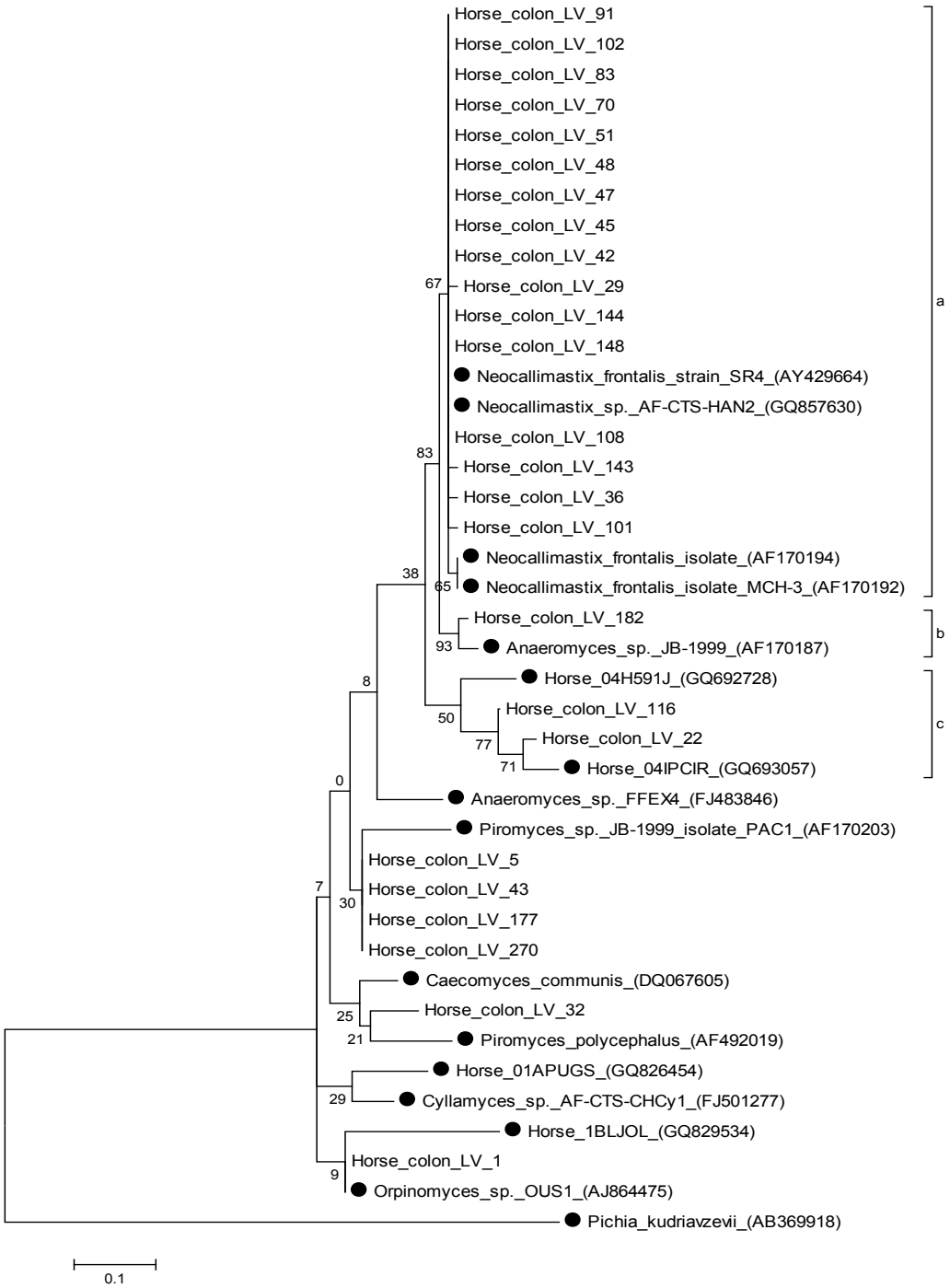

Figure 13 - Phylogenetic tree of anaerobic fungi sequences found in the left ventral colon (LVC) and analyzed in this study. Reference sequences of anaerobic fungi (with black dots) were retrieved from GenBank and phylogenetically compared with 25 sequences generated in this study and representing different OTUs. The tree was constructed using nearest–neighbor Interchange (NNI) ML heuristic method and maximum likelihood statistic method. Bootstrap values are based on 1000 replicates. **a.** Neocallimastix cluster; **b.** Anaeromyces cluster; **c**. NG1 cluster. The corresponding ITS1 and partial 5.8S region of the Ascomycetous *Pichia kudriavzevii* was used as outgroup. GenBank accession numbers of reference sequences are given in parentheses.

5.6 The anaerobic fungi of the left dorsal colon (LDC) content

In total, 253 clones of *E.coli* were randomly selected from LDC ITS1 clone library and their plasmid DNA was checked for the presence of ITS1 inserts. 49% of the clones were either false positive or contained too short inserts, 51% of the samples bore plasmid with inserts of proper length. Their sequences were used for the phylogenetic analysis.

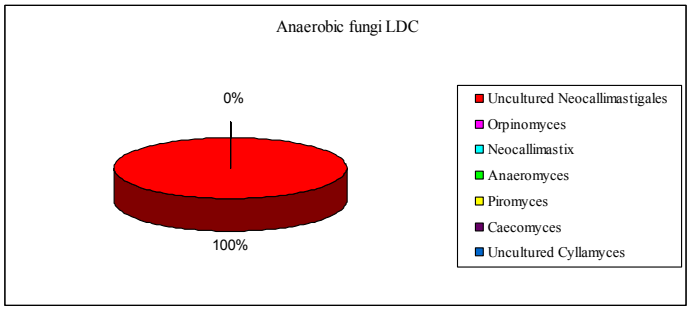

Figure 14 - Overview of the classification of all ITS1 sequences of anaerobic fungi found in the left dorsal colon (LDC) in this study based on the BLAST search.

128 new ITS sequences (complete ITS1 and partial 5.8S rDNA) were generated from total genomic DNA of intestinal content of the left dorsal colon. All the sequences were compared to the GenBank nucleotide database using the BLAST search facility. The alignment was performed in the same way with the same reference strains as in the paragraph 5.3. 23 sequences generated in this study, representing different operational taxonomic units (OTU), were selected and included in the phylogenetic tree illustrated in the figure 16.

Based on BLAST determination 100% fungal of the sequences generated from the left dorsal colon digesta have been affiliated with high identity (>98%) with uncultured Neocallimastigales. No sequences were assigned to any of the six know genera of anaerobic fungi (Fig. 14).

The comparison of uncultured Neocallimastigales clones generated in this study from the LDC content with the new groups of uncultured Neocallimastigales described by Liggenstoffer et al. (2010) in the horse feces is shown in the figure 15. 58% of ITS1 sequences from our LVC library (73 clones) were closely related to the group NG1 (cluster **a** in the figure 16). No similarities were found with sequences of the groups NG2 and NG3. The remaining 42% of our sequences did not cluster with any of known genera of anaerobic fungi. In the phylogenetic tree (Fig. 16) these unknown uncultured gut fungi create well supported consistent group described as cluster **b**, which is quite distant from the core of reference strains of anaerobic fungi.

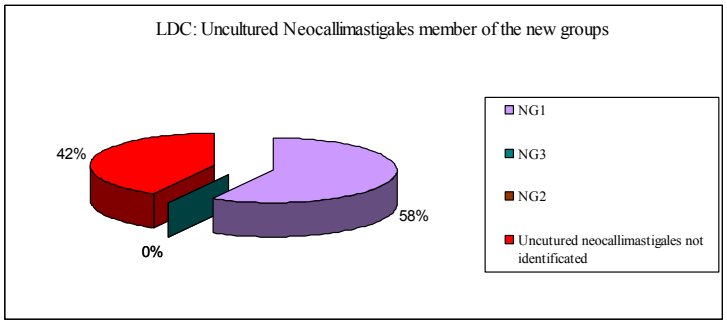

Figure 15 - Overview of similarity of LDC sequences of uncultured Neocallimastigales (100% of the total sequence encountered in the LDC in this study) with sequences of the new groups NG1, NG2 and NG3.

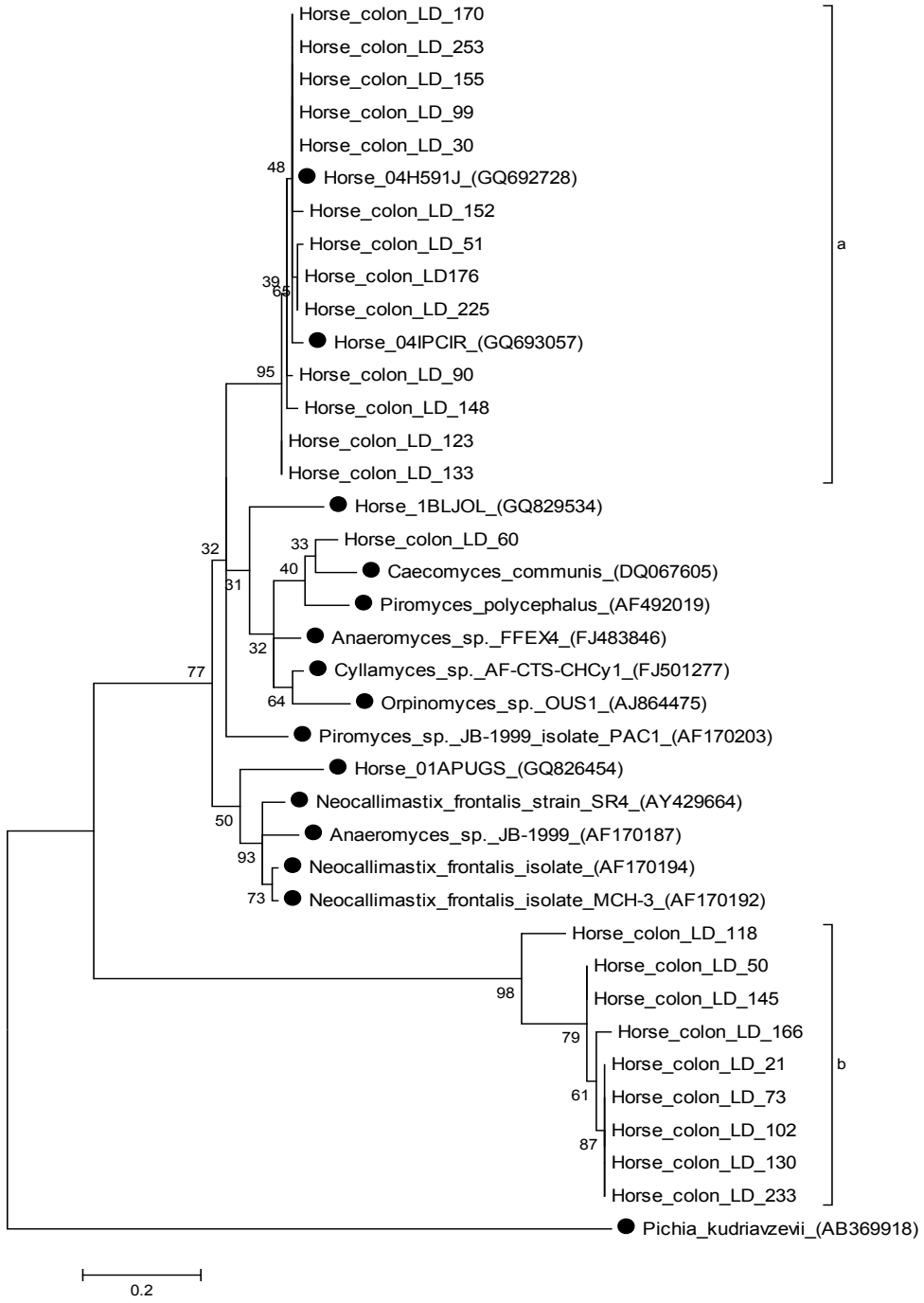

Figure 16 - Phylogenetic tree of anaerobic fungi sequences found in the left dorsal colon (LDC) and analyzed in this study. Reference sequences of anaerobic fungi (with black dots) were retrieved from GenBank and phylogenetically compared with 23 sequences generated in this study. The tree was constructed using nearest–neighbor Interchange (NNI) ML heuristic method and maximum likelihood statistic method. Bootstrap values are based on 1000 replicates. **a**. NG1 cluster; **b.** cluster of unknown anaerobic fungi. The corresponding ITS1 and partial 5.8S region of the Ascomycetous *Pichia kudriavzevii* was used as outgroup. GenBank accession numbers of reference sequences are given in parentheses.

5.7 The anaerobic fungi of the right dorsal colon (RDC) content

In total, 301 clones of *E.coli* were randomly selected from the right dorsal colon ITS1 clone library and their plasmid DNA was checked for the presence of ITS1 inserts. 37% of the clones were either false positive or contained too short inserts. 63% of the samples bore plasmid with inserts of proper length and their sequences wee used for phylogenetic study.

189 new ITS sequences (complete ITS1 and partial 5.8S rDNA) were generated from the total genomic DNA of the content of the right dorsal colon. All the sequences were compared to the GenBank nucleotide database using the BLAST search facility. The alignment was performed in the same way with the same reference strains as in the paragraph 5.3. 33 sequences generated in this study were selected and included in the phylogenetic tree illustrated in the figure 19.

Based on BLAST determination all 189 fungal sequences generated from the right dorsal colon digesta have been assigned to the order Neocallimastigales, 185 of these clones with high identity (97-100%) and only 4 clones with identity ranging from 94% up to 96%. 88% of the sequences were affiliated again with uncultured Neocallimastigales. 5% of the total sequences were assigned to the genus *Neocallimastix*, 3% to the genus *Orpinomyces* and 1% to the genus *Anaeromyces*. 3% of the sequences were affiliated with uncultured *Cyllamyces*. No sequences were affiliated with *Piromyces, Caecomyces*, and cultured *Cyllamyces*. These data are graphically depicted in the figure 17.

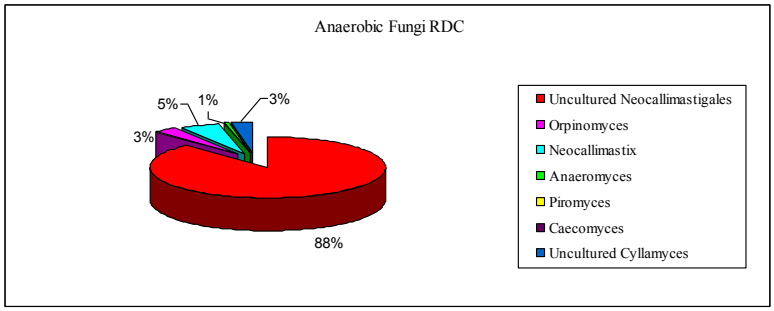

Figure 17 - Overview of the classification of all ITS1 sequences of anaerobic fungi found in the right dorsal colon (RDC) in this study based on the BLAST search.

The comparison of 166 uncultured Neocallimastigales clones generated in this study from the RDC content with the new groups of uncultured Neocallimastigales described by Liggenstoffer et al. (2010) in the horse feces is shown in the figure 18. 27% of ITS1 sequences from our RDC library (44 clones) were related to the group NG1 (cluster **a** in the figure 19). No similarities were found with the sequences of the groups NG2 and NG3. The remaining 73% of our sequences did not cluster with any of known genera of anaerobic fungi. In the phylogenetic tree (Fig. 19) these unknown, uncultured, gut fungi create relatively well supported consistent group described as cluster **d.**

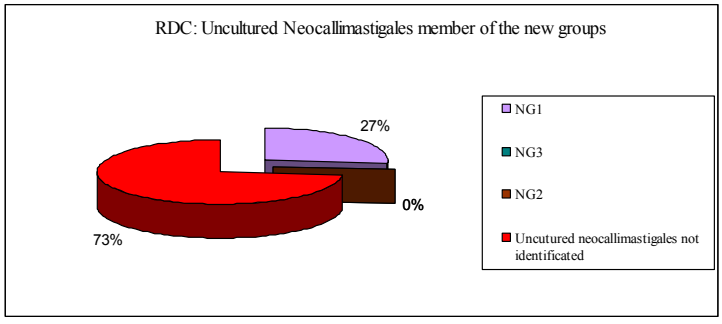

Figure 18 - Overview of similarity of RDC sequences of uncultured Neocallimastigales (88% of the total sequence encountered in the RDC in this study) with sequences of the new groups NG1, NG2 and NG3.

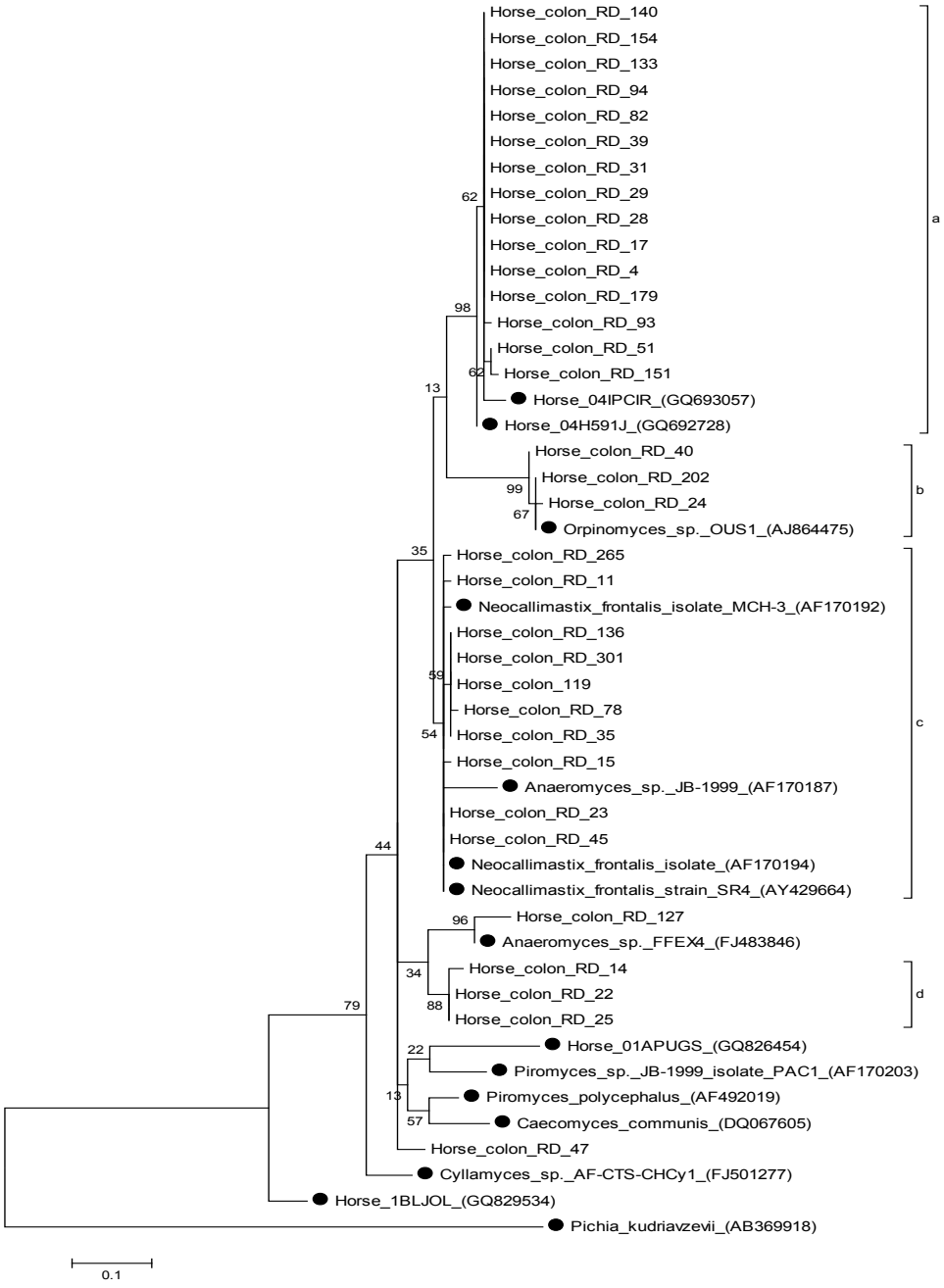

Figure 19 - Phylogenetic tree of anaerobic fungi sequences found in the right dorsal colon (RDC) and analyzed in this study. Reference sequences of anaerobic fungi (with black dots) were retrieved from GenBank and phylogenetically compared with 23 sequences generated in this study. The tree was constructed using nearest–neighbor Interchange (NNI) ML heuristic method and maximum likelihood statistic method. Bootstrap values are based on 1000 replicates. **a.** NG1 cluster; **b.** Orpinomyces-like cluster ; **c.** Neocallimastix-like cluster; d. cluster of unknown anaerobic fungi. The corresponding ITS1 and partial 5.8S region of the Ascomycetous *Pichia kudriavzevii* was used as outgroup. GenBank accession numbers of reference sequences are given in parentheses.

5.8 The anaerobic fungi of the rectal content (feces)

In total, 161 clones of *E.coli* were randomly selected from the rectal content ITS1 clone library and their plasmid DNA was checked for the presence of ITS1 inserts. 43% of the clones were either false positive or contained too short inserts. 57% of the samples bore plasmid with the inserts of the proper length and their sequences wee used for phylogenetic study.

92 new ITS sequences (complete ITS1 and partial 5.8S rDNA) were generated from the total genomic DNA of content of horse rectum. All the sequences were compared to the GenBank nucleotide database using the BLAST search facility. The alignment was performed in the same way with the same reference strains as in the paragraph 5.3. 35 sequences generated in this study were selected and included in the phylogenetic tree illustrated in figure 22.

Based on BLAST determination all the 92 fungal sequences generated from the rectal content digesta have been assigned to the order Neocallimastigales, 77 clones with high identity (97-100%) and 15 clones with identity ranging from 94% up to 96%. The majority of the sequences (77%) was classified as *Neocallimastix*. 2% of the sequences were assigned to the genus *Orpinomyces.* 5% of the samples were affiliated with high identity 97% – 100% with the uncultured *Neocallimastigales* and 16% with the uncultured *Cyllamyces*, with identity 94% - 96% (no sequences had identity >98% with *Cyllamyces*). No sequences were affiliated with *Piromyces, Caecomyces*, and cultured *Cyllamyces.* These data are graphically depicted in the figure 20.

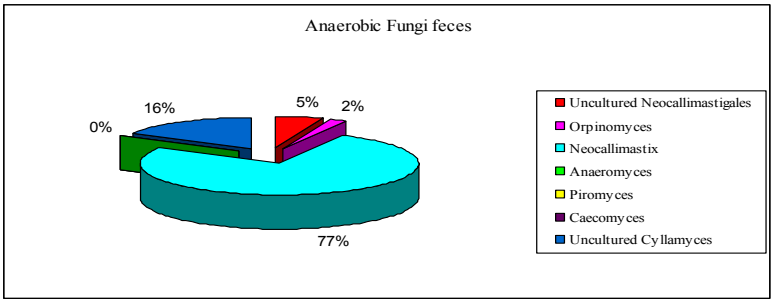

Figure 20 - Overview of the classification of all ITS1 sequences of anaerobic fungi found in the feces in this study based on the BLAST search.

The comparison of 5% (5 clones) of uncultured Neocallimastigales clones generated in this study from the feces with the new groups of the uncultured Neocallimastigales described by Liggenstoffer et al. (2010) in horse feces is shown in the figure 21. 40% of ITS1 sequences from our rectal digesta library (2 clones) were related to the group NG1 (cluster **c** in the phylogenetic tree in the figure 22). No similarities were found with the sequences of the groups NG2 and NG3. The remaining 60% (3 clones only) of our sequences did not cluster with any of known genera of anaerobic fungi.

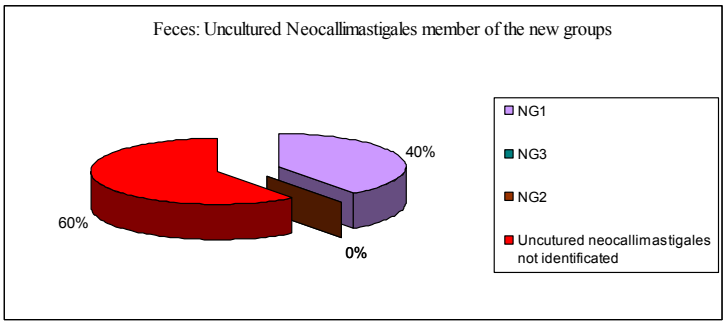

Figure 21 - Overview of similarity of feces sequences of uncultured Neocallimastigales (5% of the total sequence encountered in the rectum in this study) with sequences of the new groups NG1, NG2 and NG3.

The phylogenetic analysis depicted in the figure 22 however shows that, the relationship among the fungal sequences from the feces is complex and the differentiation between *Neocallimastix* strains and the uncultured *Cyllamyces* strains is not unambiguous. The sequences identified by BLAST search as *Neocallimastix* are split into two clusters: *Neocallimastix frontalis* cluster **a**, and *Neocallimastix* cluster **c** 32% of *Neocallimastix* sp. strains represented in the tree by the clones 43, 37, 100, 105, 63, 79, 125, 99 and 106). The sequences identified by BLAST search as uncultured *Cyllamyces* are also split into two clusters: cluster **b** (clones 53, 54, 71) is embedded between the *Neocallimastix* groups **a** and **c**; cluster **d** (clones 27, 93) representing the second part of uncultured *Cyllamyces* is embedded inside the complex mixture of reference strains; the *Orpinomyces* cluster **e** (clones 28, 144) is very well supported.

Figure 22 - Phylogenetic tree of anaerobic fungi sequences found in the feces and analyzed in this study. Reference sequences of anaerobic fungi (with black dots) were retrieved from GenBank and phylogenetically compared with 35 sequences generated in this study. The tree was constructed using nearest–neighbor Interchange (NNI) ML heuristic method and maximum likelihood statistic method. Bootstrap values are based on 1000 replicates. **a.** Neocallimastix cluster; **b**. Uncultured Cyllamyces I cluster; **c** Neocallimastix-like cluster; **d.** Uncultured Cyllamyces II cluster; **e**. Orpinomyces cluster. The corresponding ITS1 and partial 5.8S region of the Ascomycetous *Pichia kudriavzevii* was used as outgroup. GenBank accession numbers of reference sequences are given in parentheses.

5.9 The anaerobic fungi of the horse hindgut

The phylogenetic analysis of the anaerobic fungi diversity along the horse digestive tract showed interesting differences in the six studied parts of the equine hindgut. The uncultured Neocallimastigales represent the majority of anaerobic fungi (81%) described in this work in the horse hindgut, as shown in the figure 23. However, their distribution along the digestive tract is not regular, as shown in the figure 24, that summarizes the incidence of the different anaerobic fungi, in each part of the horse hindgut. It is evident that, the occurrence of the anaerobic fungi species is quite different in the left ventral colon and very different in the feces. The left ventral colon is also specific with respect to the uncultured Neocallimastigales, because LVC is occupied by majority of the new unknown anaerobic fungi. The other parts of the hindgut have significant proportion of uncultured Neocallimastigales of NG1 group described by Liggenstoffer et al. (2010) in the horse feces (Fig. 25).

The cluster analysis, representing the statistical evaluation of the anaerobic fungi communities correlation among the different parts of the horse hindgut, is shown in the figure 26. These results indicate a certain similarity of the fungal microbial population of the cecum with right dorsal colon (40%) and a lower population similarity of left ventral colon and feces (22%). The fungal community of the right ventral and left dorsal colon is very similar.

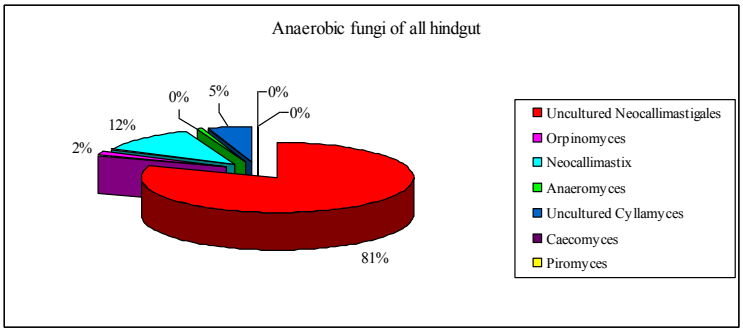

Figure 23 - Overview of classification of all ITS1 sequences of anaerobic fungi found in the horse hindgut in this study based on the BLAST search.

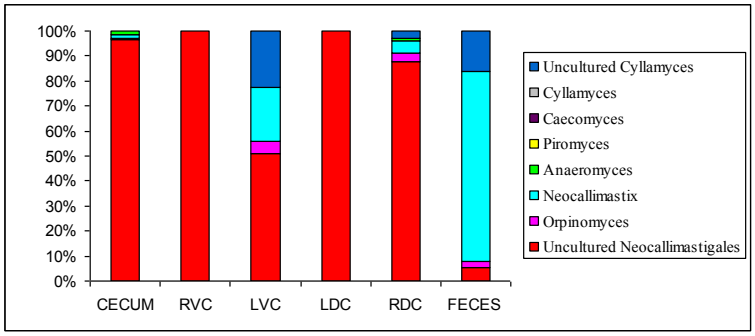

Figure 24 - Distribution of ITS1 sequences of anaerobic fungi in each part of the horse hindgut.

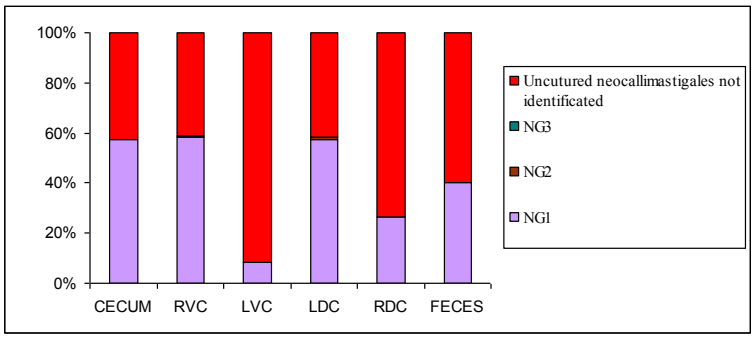

Figure 25 - Occurrence of uncultured anaerobic fungi affiliated with the group NG1 in each part of the horse hindgut.

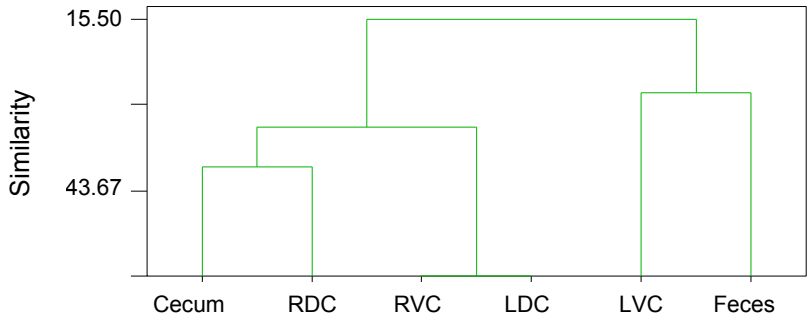

Figure 26 - Similarities among communities of anaerobic fungi of the different parts of horse hindgut. Statistical evaluation was calculated by the Pearson correlation coefficient and grouping was performed by hierarchical cluster analysis using MINITAB 12.1.

The figure 27 shows the phylogenetic tree constructed from 65 representative sequences from all the studied parts of horse digestive tract. The red branch contains the uncultured Neocallimastigales similar to the NG1 new group. These sequences, forming a compact cluster, were found in each part of the digestive tract and they represent the most important part of equine fungal population. The pink branch contains the sequences of uncultured Neocallimastigales and the sequences of the related to the genus *Neocallimastix.* These sequences, forming also a compact cluster, were found in the cecum, right dorsal (RDC) and left ventral colon (LVC) and feces. Despite the big number of uncultured Neocallimastigales found in the right ventral (RVC) and left dorsal colon (LDC), sequences of Neocallimastix species were not found in these two studied parts of the hindgut. The blue branch contains the sequences of *Orpinomyces* species and uncultured Neocallimastigales found in the cecum, right dorsal (RDC) and left ventral colon (LVC) and feces and not present in the RVC and LDC. The orange branch includes the sequences from all parts of the hindgut and identifies the uncultured Neocallimastigales and uncultured *Cyllamyces*. This branch is not well supported, so the reliability of this clustering is very low. The violet branch contains the sequences found in the cecum and RDC classified by BLAST as *Anaeromyces* sp., however the relationship with the reference strain of *Anaeromyces* in this tree is not well supported. The yellow branch includes all the sequences of the uncultured Neocallimastigales,

uncultured *Cyllamyces* and *Neocallimastix* found in the cecum, right ventral (RVC), left dorsal colon (LDC) and feces. However in this tree, these sequences make a separate cluster and can be considered as a new unknown group of anaerobic fungi.

The sequences used in the phylogenetic tree construction and the nearest relative sequences from the GenBank are reported in the appendix n. 1.

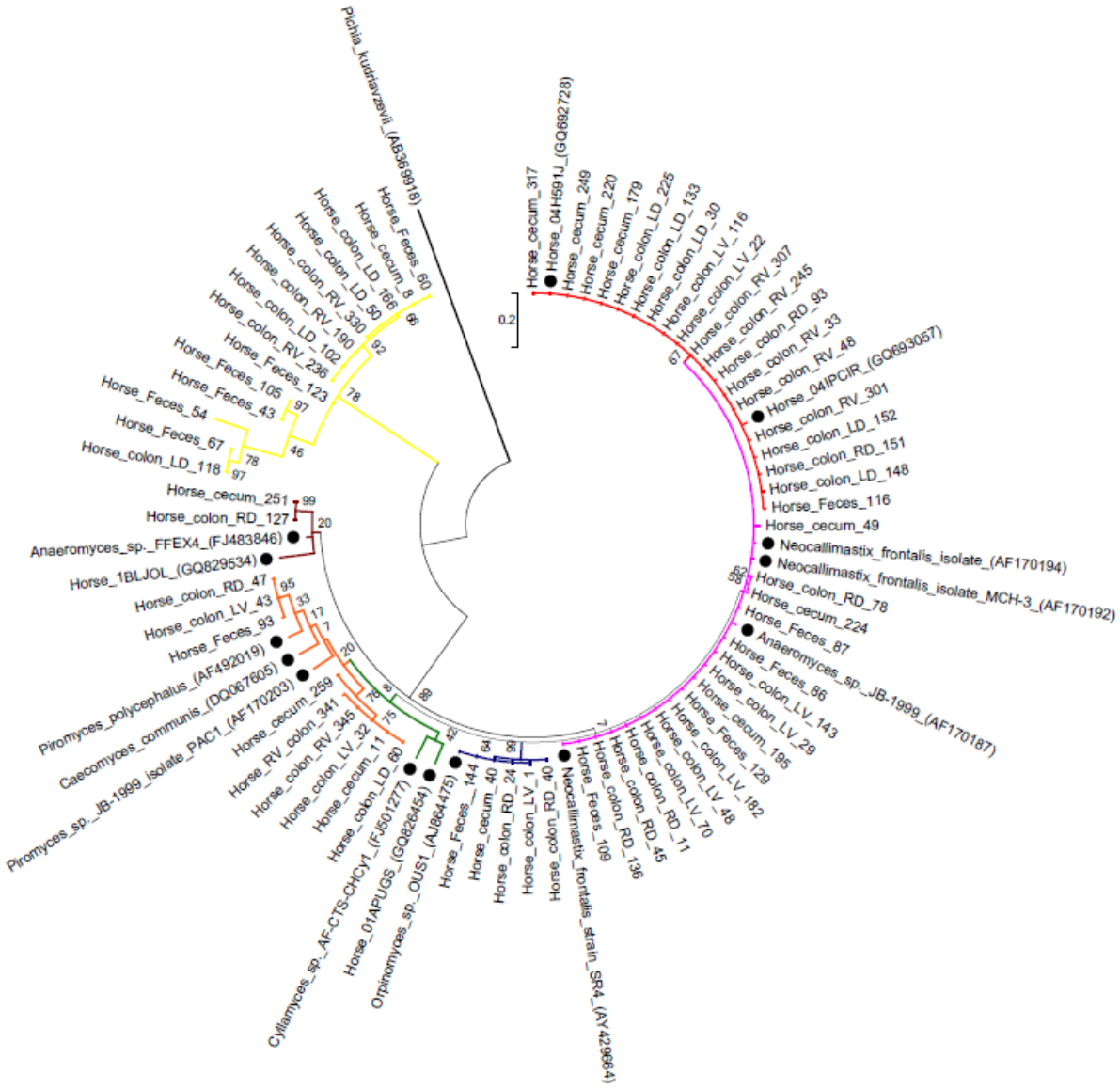

Figure 27 - Phylogenetic tree of anaerobic fungal sequences found in the horse hindgut and analyzed in this study. Reference sequences of anaerobic fungi (with black dots) were retrieved from GenBank and phylogenetically compared with 65 sequences generated in this study. The tree was constructed using nearest–neighbor Interchange (NNI) ML heuristic method and maximum likelihood statistic method. Bootstrap values are based on 1000 replicates. **Red:** NG1 cluster; **pink:** *Neocallimastix* cluster; **blue:** *Orpinomyces* cluster*;* **orange:** *Piromyces-like* cluster; **violet.** *Anaeromyces-like* cluster; **yellow:** cluster of unknown anaerobic fungi. The corresponding ITS1 and partial 5.8S region of the Ascomycetous *Pichia kudriavzevii* was used as outgroup. GenBank accession numbers of reference sequences are given in parentheses.

5.10 Discussion

The aim of this thesis was to provide information on the diversity of anaerobic fungi within the different parts of the horse lower digestive tract. There are very limited published data on the fungal, microbial population composition of the equine hindgut and most of the studies are on samples from feces. So the original and pioneer results of this work are very difficult to discuss, because of the lack of data dealing with the same topic.

The major finding of this study is the prevalence of uncultured *Neocallimastigales* in the lower digestive tract, representing 81% and the different distribution of uncultured anaerobic fungi in each part of the hindgut, ranging from 100% in RVC and LDC to 5% in the rectum (feces). The completely different diversity of gut fungi in the rectum (feces) compared to the cecum and colon is also the result of great importance. Feces are normally used as reference samples to describe the microbial population of the digestive tract (Davies et al., 1993, McGranaghan et al., 1999, Nicholson et al., 2010), however our study brings the evidence that fecal samples do not reflect the factual microbial population composition of the hindgut. A similar observation was published by Griffith et al. (2009), who compared anaerobic fungal population of rumen and feces of the same cow and found considerable differences in the abundance of the different taxa. Fungi with bulbous morphotypes (*Caecomyces* and *Cyllamyces*) were the most abundant in fresh feces, where they comprised a 5-fold greater proportion of the total fungal population than in the rumen. Conversely polycentric morphotypes (*Orpinomyces* and *Anaeromyces*) were less frequently isolated from the fresh feces rather than from the rumen digesta. Davies et al. (1993) found that the aerobic survival of anaerobic fungi increased progressively in the GI tract regions distal to the rumen and they suggested that specific aerotolerant resting structures can develop during the passage through the hindgut. In the horse feces the fungi of genus Neocallimastix were prevalent, representing 77% of the sequences, followed by uncultured Cyllamyces (16%). These results could indicate that Neocallimastix are the most aerotolerant strains, which

simplifies their isolation and explains the number of published data, resulting in the fact that Neocallimastix is the best studied and described genus among anaerobic fungi. On the other hand, 5% of uncultured Neocallimastigales in feces could indicate that these unknown strains are highly oxygen sensitive, which complicates and makes impossible their isolation. Despite the significant differences between the rectum (feces) and the other parts of the horse GI tract, in our opinion, the feces can serve as qualitative reference sample, because all species (except *Anaeromyces* detected in low amount 1-2% in cecum and RDC) found in hindgut were present in feces.

The evaluation of the results obtained from each part of horse hindgut reveals, that the fungal population of the cecum, the first and the third segments of the colon, RVC and LDC, are totally unknown and are different from the consecutive part of the LVC tract, despite that RVC and LVC are, usually, considered as a unique part of horse digestive tract. The notable difference in fungal diversity between LVC and LDC can be caused by the pelvic flexure. It divides the ventral and the dorsal colon and is responsible for selective retention of coarse particles, which means that these are retained in cecum and ventral colon and liquid and fine particles move on into the dorsal colon. This separation of bigger food particles, due to the pelvic flexure and the retropulsive movements (as reported in the chapter 1), is responsible for keeping the LVC replete. Cecum and ventral colon are therefore the sites with the highest MRT (mean retention time) of digesta (about 9 hours) and sites of the main lignocellulose degradation. In other words, microorganisms have a long time to degrade the structural polysaccharides of plant biomass and also have time for their own propagation. This can explain the increased diversity of anaerobic fungal species in LVC found in this study. 50% of the sequences detected in LVC were classified at the genus level represented by *Neocallimastix*, *Cyllamyces* and *Orpinomyces*, and "only" 50% of the sequences were assigned to unknown, uncultured, anaerobic fungi, which is half of unknown fungi detected in RVC. Crossing over the pelvic flexure, there is again a massive increase of incidence of unknown uncultured Neocallimastigales in LDC. It could be presumed that the known species of gut fungi are probably attached to bigger feed particles and do not proceed into dorsal colon in significant amount. The LDC represents the hindgut site with a short

retention time (Varloud et al., 2004) and the microbial diversity of this part definitely depends on the moment of sampling. The RDC is the site of another selective mechanism (known as the colonic separation mechanism) responsible for the prolonged retention of fluid and smaller food particles (Drogoul et al., 2000). In RDC the incidence of known species of anaerobic fungi detected in LVC was proved again (*Neocallimastix, Orpinomyces, Cyllamyces* and moreover *Anaeromyces*), however in very low amount. The explanation is that these classified species of gut fungi enter the RDC attached to small feed particles can be available. This hypothesis could be elucidated by the analysis of fluid and feed particles separately, however this experiment has not been performed in this study. From the RDC the digesta pass dorsally into the colon transversum, where the contractions of the muscular wall squeeze the content. The liquid then flows back (see figure 4 of chapter 1) into the proximal colon and digesta finish the path along the hindgut as drained, inspissated feces, containing the undigested residues. The completely different diversity of fungal population and the very low proportion of unknown gut fungi in the feces sample is not simple to explain. The dominance of Neocallimastix species in fecal samples can be possibly explained by tight attachment of mycelia to residual food particles, which protects them to be washed out. Neocallimastix species indeed produce the dense branch mycelium intergrowing inside the plant tissue on the basis of its polyflagellate zoospore ultrastructure (Heath, et al. 1983). However this is not valid for Cyllamyces strains (Ozkose, et al., 2001) which, were detected in feces also in considerable amount of 16%. Our finding of *Neocallimastix* prevalence and low proportion of uknown fungi (uncultured Neocallimastigales) in feces is moreover in contrast with the results of Liggenstoffer et al. (2010), who studied the diversity of anaerobic fungi in feces of Equidae (three horses, donkey, wild-ass, and three zebras). *Neocallimastix* species were found only in Somali wild ass in considerable amount (45%), *Caecomyces* represented 15% of the sequences in the same animal and 12% in one horse individual. However in all the other animals the absolute dominance of uncultured anaerobic fungi assigned to the new groups NG1 and NG3 was described. In our samples the proportions of the sequences similar to the NG1 group (with respect to the total anaerobic fungal community) were detected in high amount in the cecum

(55%), RVC (58%), LDC (57%), and RDC (23%), but in low amount in the LVC (4%) and feces (2%). This results again demonstrate the similarty of fungal diversity in the left ventral colon and rectum (feces).

This similarity, between the LVC and feces, was observed also when the bacterial and archeael population profile was analysed in the same samples using the DGGE method (Denaturating Gradient Gel Electrophoresis) as described by Mura et al. (2013). Monitoring the bacterial community structure using 16S rDNA gene fragments indicated the significant differences among bacterial profiles of cecum, left ventral colon (LVC) and dorsal colon and similarity of LVC with feces. Monitoring of archaeal community structure (also 16S rDNA gene fragments) indicated the different profile in cecum, similarity of RVC, LDC and RDC, and again the similarity of LVC with feces (see figure 28 and 29).

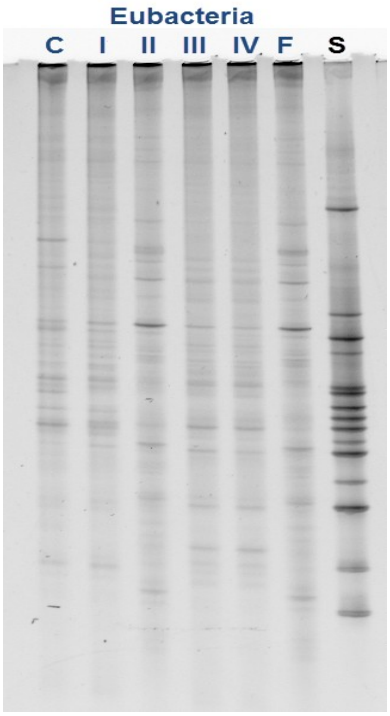

Figure 28 - DGGE profile for Eubacteria in the different tract of hindgut. **S**. standard; **C.** cecum; **I.** RVC; **II.** LVC; **III.** LDC; **IV**. RDC. **F**. feces.

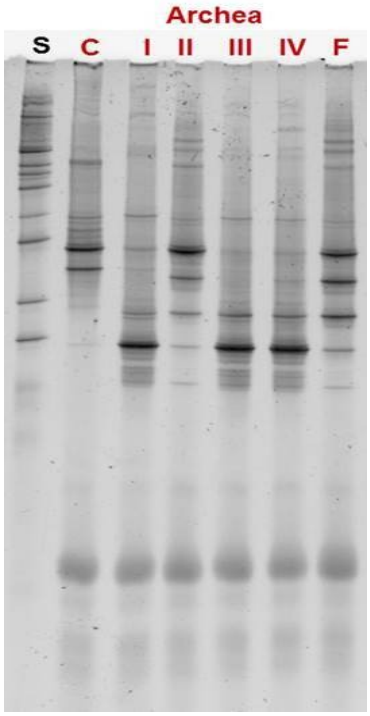

Figure 29 - DGGE profile for Archea in the different tract of hindgut. **S**. standard; **C.** cecum; **I.** RVC; **II.** LVC; **III.** LDC; **IV**. RDC. **F**. feces.

The comparison of the sequences retrieved in this study with GenBank data also indicates the similarity of LVC and feces. Horse fungal sequences from the LVC were mostly similar to those obtained from ruminants, especially cow and bison and only 4 sequences had high identity with horse and zebra sequences. The same is valid for fecal sequences with high identity shared with yak and cow sequences and only 4 ITS1 fragments are similar to those obtained from zebra and ass. On the other hand, the sequences amplified from the other parts are typical for Equidae. Most sequences from cecum (139) are similar or identical with those from zebra (93 sequences) and horse (45 sequences). "Equidae" fungal sequences are massively prevalent also in RVC and LDC parts. Also in RDC the "Equidae" sequences created majority (123 sequences with high identity with horse and zebra sequences), however ITS fragments similar to cow and bison sequences are present in lower amount (51 sequences).

These results indicate the dissimilar fermentation activities in the different segments of horse lower gastrointestinal tract. The hindgut thus cannot be considered as an unique tract but, each single segment represents a different fermentative chamber with its own microbial population. The enormous large number of unknown, uncultured species of anaerobic fungi however makes very difficult the evaluation of the results. 81% of the sequences of equine gut fungi recovered in this study do not correspond to any previous, characterised species, therefore there are no information about their physiology, biochemistry and hydrolytic activities.

The data presented here provide an important first step in indicating the considerable uncharacterised fungal diversity of the equine gut. The large amount of unknown sequences suggests that there may be new groups of gut fungi, which may be equine only. The sequences identified here allow for the design of specific oligonucleotides probes, which can be used to screen for the potential presence/absence of various phylo-groups and will provide convenient method for monitoring the effect of various known modulators as dietary changes, stress, exercise, age, drug treatment or disease on the equine microflora for a better understanding the relationship between the microbial composition and the host physiology or pathophysiology.

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Conclusions

The molecular, biological analysis of fungal internal transcribed spacer (ITS) of ribosomal DNA amplified from samples of digesta has shown a considerable diversity in the anaerobic fungal population found in the different segments of the horse hindgut and considerable diversity comparing with the feces. The results presented in this study indicate the dissimilar fermentation activities in different segments of horse lower gastrointestinal tract. The hindgut thus cannot be considered as a unique compartment, but each single segment represents a different fermentative chamber with a different microbial population. The enormous large number of unknown, uncultured species of anaerobic fungi however makes very difficult the evaluation of our the results. Nevertheless the following conclusions can be derived from the results obtained in this study.

1. The horse digestive tract is populated by unknown, not yet cultivated species of anaerobic fungi of class Neocallimastigomycetes (81%). This gap can be overcome only by the effort to cultivate new fungal species from horse hindgut.

2. Only a limited percentage of identified sequences can be classified into known species of gut fungi (19 %).

3. A new cluster of anaerobic fungi was found in the horse digestive tract.

4. The conventional microbiological cultivation techniques underestimate the diversity of the anaerobic fungal population in horse hindgut.

5. The gut fungi from horse cecum, right ventral and left dorsal colon seem to be specific for the Equidae family.

6. The gut fungi from horse left ventral colon and rectum are similar to the rumen anaerobic fungi found in cattle and bison.

7. Different segments of horse hindgut are populated by different species of anaerobic fungi, in particular rectum (feces) differ considerably in fungal diversity compared with the cecum and three colon tracts (RVC, LDC and RDC). Consequently the results fecal samples are not representative of the microbial population of the horse large intestine.

Appendix 1.

In the following table the sequences used in the phylogenetic tree construction and the nearest relative sequences from the GenBank are reported.

Table 1 – Sequences number used in the phylogenetic trees construction. Similarity with the GenBank sequences : Red: 96-93% ; Black: 100-97%.

	CECUM	RVC	LVC	LDC	RDC	FECES
AJ864475	C141					F28
AJ429664						F106 F117 F123
AY997064					RDC136	F87 F99 F102
FJ501295			LVC1		RDC24 RDC31 RDC40 RDC202	F144
GQ604950				LDC170		
GQ605287				LDC152		
GQ686303	C11					
GQ686305				LDC60		F67
GQ689438	C259	RVC341 RVC345	LVC32	LDC118	RDC14 RDC22 RDC25	
GQ693478	C317					
GQ693598				LDC30	RDC82	
GQ696236	C40					
GQ757158			LVC91			
GQ782694	C38					
GQ785309					RDC29	

GQ785384	C249					
GQ785750	C179 C216	RVC48 RVC236			RDC154	
GQ787984				LDC176		
GQ788927				LDC123, LDC133		
GQ800614		RVC301	LVC22	LDC21 LDC130 LDC253	RDC39	
GQ806846		RVC252				
GQ807426					RDC4	
GQ811038					RDC28	
GQ811925					RDC51, RDC151	
GQ812715					RDC179	
GQ813691		RVC292				
GQ817333		RVC166		LDC73		
GQ818101	C13 C220 C275	RVC82 RVC171 RVC307		LDC50	RDC17 RDC133	F60-F116
GQ823602		RVC33				
GQ823722				LDC166		
GQ835558		RVC 293				
GQ835716				LDC102	RDC93	
GQ835874		RVC109		LDC90 LDC148	RDC140	
GQ836160		RVC330				
GQ836227	C72	RVC190		LDC99		

				LDC233		
GQ836353		RVC245			RDC94	
GQ836403	C8-C267			LDC51 LDC225		
GQ839500				LDC145		
GQ842090		RVC122				
GQ840318		RVC225				
GQ843180				LDC155		
GQ850312			LVC5 LVC48 LVC43 LVC177 LVC270			F27 F53 F54 F71 F93
GQ850373					RDC47	
GQ857630	C195 C309				RDC119 RDC301	F21 F43 F86 F109
GU910843	C49 C85 C173 C224		LVC36 LVC42 LVC47 LVC83 LVC101 LVC102		RDC15 RDC23 RDC35 RDC265	
GU910869					RDC45	
GU910896			LVC29 LVC143 LVC144 LVC148		RDC78	

			LVC108			
GU911073					RDC 11	
HQ585902			LVC182			
JN560945	C251				RDC127	
JQ782544						F30 F56 F63 F125
JQ782546						F37 F58 F84 F97 F100 F115 F129 F152
JQ782549						F66 F79 F105

AJ864475

```
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                     /mol_type="genomic DNA"
                     /isolate="OUS1"
                     /db_xref="taxon:301046"
                     /clone="V5-1"
     gene            <1..841
                     /gene="18S rRNA"
     rRNA            <1..841
                     /gene="18S rRNA"
                     /product="18S ribosomal RNA"
     misc feature    842..1056
                     /note="internal transcribed spacer 1, ITS1"
     gene            1057..1241
                     /gene="5.8S rRNA"
     rRNA            1057..1241
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     misc feature    1242..1418
                     /note="internal transcribed spacer 2, ITS2"
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     rRNA            1419..>4868
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                     /product="28S ribosomal RNA"
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AY429664

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FEATURES             Location/Qualifiers
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                     /clone="1"
     rRNA            <1..9
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                    /product="18S ribosomal RNA"
     misc_RNA       10..275
                    /product="internal transcribed spacer 1"
     rRNA           276..418
                    /product="5.8S ribosomal RNA"
     misc_RNA       419..638
                    /product="internal transcribed spacer 2"
     rRNA           639..>679
                    /product="28S ribosomal RNA"
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AY997064

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FEATURES            Location/Qualifiers
     source         1..637
                    /organism="Neocallimastix sp. GE13"
                    /mol_type="genomic DNA"
                    /strain="GE13 (G. W. Griffith)"
                    /isolate="AFTOL-ID 638"
                    /db_xref="AFTOL:638"
                    /db_xref="taxon:324672"
     misc_RNA       <1..265
                    /product="internal transcribed spacer 1"
     rRNA           266..424
                    /product="5.8S ribosomal RNA"
     misc_RNA       425..>637
                    /product="internal transcribed spacer 2"
```

FJ501295

```
FEATURES             Location/Qualifiers
     source          1..710
                     /organism="Orpinomyces sp. AF-CTS-BTO2"
                     /mol_type="genomic DNA"
                     /isolate="AF-CTS-BTO2"
                     /isolation_source="rumen fluid"
                     /host="cross bred steers"
                     /db_xref="taxon:579089"
     misc RNA        <1..>710
                     /note="contains 18S ribosomal RNA, internal
transcribed
                     spacer 1, 5.8S ribosomal RNA, and internal
transcribed
                     spacer 2"
```

GQ604950

```
FEATURES             Location/Qualifiers
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                     /mol_type="genomic DNA"
                     /isolation_source="feces"
                     /host="Pere David's deer"
                     /db_xref="taxon:325898"
                     /clone="PereDavidsDeer02EXM5T"
                     /environmental_sample
                     /country="USA: Oklahoma City Zoo"
     misc RNA        <1..>228
                     /product="internal transcribed spacer 1"
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GQ605287

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                     /mol_type="genomic DNA"
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/host="Pere David's deer"
/db_xref="taxon:325898"
/clone="PereDavidsDeer02EB1RO"
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misc_RNA <1..>229
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GQ686303

FEATURES Location/Qualifiers
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/mol_type="genomic DNA"
/isolation_source="feces"
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/environmental_sample
/country="USA: Oklahoma City Zoo"
misc_RNA <1..>256
/product="internal transcribed spacer 1"

GQ686305

FEATURES Location/Qualifiers
source 1..255
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/mol_type="genomic DNA"
/isolation_source="feces"
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/db_xref="taxon:325898"
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/environmental_sample
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misc_RNA <1..>255
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GQ689438

```
FEATURES             Location/Qualifiers
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                     /mol_type="genomic DNA"
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(locally
                     owned)"
                     /host="horse"
                     /db_xref="taxon:325898"
                     /clone="HorseTopper04IU22Y"
                     /environmental_sample
                     /country="USA: Stillwater, OK"
     misc_RNA        <1..>255
                     /product="internal transcribed spacer 1"
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GQ693478

```
FEATURES             Location/Qualifiers
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(locally
                     owned)"
                     /host="horse"
                     /db_xref="taxon:325898"
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     misc_RNA        <1..>228
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GQ693598

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(locally
                     owned)"
                     /host="horse"
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                     /environmental_sample
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                       /product="internal transcribed spacer 1"
```

GQ696236

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FEATURES             Location/Qualifiers
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                     /host="American bison"
                     /db_xref="taxon:325898"
                     /clone="AmericanBison02CZBZB"
                     /environmental_sample
                     /country="USA: Oklahoma City Zoo"
     misc RNA        <1..>220
                     /product="internal transcribed spacer 1"
```

GQ757158

FEATURES Location/Qualifiers
source 1..274
/organism="uncultured Neocallimastigales"
/mol_type="genomic DNA"
/isolation_source="feces"
/host="bontebok"
/db_xref="taxon:325898"
/clone="Bontebok02DT46X"
/environmental_sample
/country="USA: Oklahoma City Zoo"
misc_RNA <1..>274
/product="internal transcribed spacer 1"

GQ782694

FEATURES Location/Qualifiers
source 1..229
/organism="uncultured Neocallimastigales"
/mol_type="genomic DNA"
/isolation_source="feces"
/host="Grant's gazelle"
/db_xref="taxon:325898"
/clone="GrantsGazelle02DQWL7"
/environmental_sample
/country="USA: Oklahoma City Zoo"
misc_RNA <1..>229
/product="internal transcribed spacer 1"

GQ785309

FEATURES Location/Qualifiers
source 1..229
/organism="uncultured Neocallimastigales"
/mol_type="genomic DNA"
/isolation_source="feces"
/host="Grant's zebra"
/db_xref="taxon:325898"

/clone="GrantsZebra01BH02L"
/environmental_sample
/country="USA: Oklahoma City Zoo"
misc RNA <1..>229
/product="internal transcribed spacer 1"

GQ785384

FEATURES Location/Qualifiers
source 1..229
/organism="uncultured Neocallimastigales"
/mol_type="genomic DNA"
/isolation_source="feces"
/host="Grant's zebra"
/db_xref="taxon:325898"
/clone="GrantsZebra01CIC8U"
/environmental_sample
/country="USA: Oklahoma City Zoo"
misc RNA <1..>229
/product="internal transcribed spacer 1"

GQ785750

FEATURES Location/Qualifiers
source 1..230
/organism="uncultured Neocallimastigales"
/mol_type="genomic DNA"
/isolation_source="feces"
/host="Grant's zebra"
/db_xref="taxon:325898"
/clone="GrantsZebra01AZCJC"
/environmental_sample
/country="USA: Oklahoma City Zoo"
misc_RNA <1..>230
/product="internal transcribed spacer 1"

GQ787984

```
FEATURES             Location/Qualifiers
     source          1..230
                     /organism="uncultured Neocallimastigales"
                     /mol_type="genomic DNA"
                     /isolation_source="feces from individual 1"
                     /host="Grevy's zebra"
                     /db_xref="taxon:325898"
                     /clone="GrevyZebraA04IK0HM"
                     /environmental_sample
                     /country="USA: Oklahoma City Zoo"
     misc_RNA        <1..>230
                     /product="internal transcribed spacer 1"
```

GQ788927

```
FEATURES             Location/Qualifiers
     source          1..226
                     /organism="uncultured Neocallimastigales"
                     /mol_type="genomic DNA"
                     /isolation_source="feces"
                     /host="Grant's zebra"
                     /db_xref="taxon:325898"
                     /clone="GrantsZebra01BZ5C2"
                     /environmental_sample
                     /country="USA: Oklahoma City Zoo"
     misc_RNA        <1..>226
                     /product="internal transcribed spacer 1"
```

GQ800614

```
FEATURES             Location/Qualifiers
     source          1..229
                     /organism="uncultured Neocallimastigales"
                     /mol_type="genomic DNA"
                     /isolation_source="feces from individual 1"
```

/host="Grevy's zebra"
/db_xref="taxon:325898"
/clone="GrevyZebraA01CANBK"
/environmental_sample
/country="USA: Oklahoma City Zoo"
misc_RNA <1..>229
/product="internal transcribed spacer 1"

GQ807426

FEATURES Location/Qualifiers
source 1..229
/organism="uncultured Neocallimastigales"
/mol_type="genomic DNA"
/isolation_source="feces from individual 1"
/host="Grevy's zebra"
/db_xref="taxon:325898"
/clone="GrevyZebraA01BV3PL"
/environmental_sample
/country="USA: Oklahoma City Zoo"
misc_RNA <1..>229
/product="internal transcribed spacer 1"

GQ811038

FEATURES Location/Qualifiers
source 1..229
/organism="uncultured Neocallimastigales"
/mol_type="genomic DNA"
/isolation_source="feces from individual 1"
/host="Grevy's zebra"
/db_xref="taxon:325898"
/clone="GrevyZebraA04H2Z4Q"
/environmental_sample
/country="USA: Oklahoma City Zoo"
misc_RNA <1..>229
/product="internal transcribed spacer 1"

GQ811925

```
FEATURES             Location/Qualifiers
     source          1..226
                     /organism="uncultured Neocallimastigales"
                     /mol_type="genomic DNA"
                     /isolation_source="feces from individual 3"
                     /host="Grevy's zebra"
                     /db_xref="taxon:325898"
                     /clone="GrevyZebraB01BVS8Z"
                     /environmental_sample
                     /country="USA: Oklahoma City Zoo"
     misc_RNA        <1..>226
                     /product="internal transcribed spacer 1"
```

GQ812715

```
FEATURES             Location/Qualifiers
     source          1..229
                     /organism="uncultured Neocallimastigales"
                     /mol_type="genomic DNA"
                     /isolation_source="feces from individual 3"
                     /host="Grevy's zebra"
                     /db_xref="taxon:325898"
                     /clone="GrevyZebraB01BIDY9"
                     /environmental_sample
                     /country="USA: Oklahoma City Zoo"
     misc_RNA        <1..>229
                     /product="internal transcribed spacer 1"
```

GQ813691

```
FEATURES             Location/Qualifiers
     source          1..230
                     /organism="uncultured Neocallimastigales"
                     /mol_type="genomic DNA"
                     /isolation_source="feces from individual 3"
                     /host="Grevy's zebra"
                     /db_xref="taxon:325898"
                     /clone="GrevyZebraB01BBIMC"
                     /environmental_sample
                     /country="USA: Oklahoma City Zoo"
     misc_RNA        <1..>230
```

/product="internal transcribed spacer 1

GQ817333

FEATURES Location/Qualifiers
source 1..231
/organism="uncultured Neocallimastigales"
/mol_type="genomic DNA"
/isolation_source="feces from individual 3"
/host="Grevy's zebra"
/db_xref="taxon:325898"
/clone="GrevyZebraB01A6DYA"
/environmental_sample
/country="USA: Oklahoma City Zoo"
misc RNA <1..>231
/product="internal transcribed spacer 1"

GQ818101

FEATURES Location/Qualifiers
source 1..228
/organism="uncultured Neocallimastigales"
/mol_type="genomic DNA"
/isolation_source="feces from individual 3"
/host="Grevy's zebra"
/db_xref="taxon:325898"
/clone="GrevyZebraB01B8K8B"
/environmental_sample
/country="USA: Oklahoma City Zoo"
misc RNA <1..>228
/product="internal transcribed spacer 1"

GQ823602

FEATURES Location/Qualifiers
source 1..229
/organism="uncultured Neocallimastigales"
/mol_type="genomic DNA"
/isolation_source="feces"
/host="Indian hog deer"

```
                    /db_xref="taxon:325898"
                    /clone="HogDeer02DWJ10"
                    /environmental_sample
                    /country="USA: Oklahoma City Zoo"
     misc_RNA       <1..>229
                    /product="internal transcribed spacer 1"
```

GQ823722

```
FEATURES            Location/Qualifiers
     source         1..229
                    /organism="uncultured Neocallimastigales"
                    /mol_type="genomic DNA"
                    /isolation_source="feces"
                    /host="Indian hog deer"
                    /db_xref="taxon:325898"
                    /clone="HogDeer02D6BIO"
                    /environmental_sample
                    /country="USA: Oklahoma City Zoo"
     misc_RNA       <1..>229
                    /product="internal transcribed spacer 1"
```

GQ835558

```
FEATURES            Location/Qualifiers
     source         1..256
                    /organism="uncultured Neocallimastigales"
                    /mol_type="genomic DNA"
                    /isolation_source="feces from 18 year old male
horse
                    'Rio'"
                    /host="horse"
                    /db_xref="taxon:325898"
                    /clone="HorseRio01BLCNH"
                    /environmental_sample
                    /country="USA: Stillwater, OK"
     misc_RNA       <1..>256
                    /product="internal transcribed spacer 1"
```

GQ835716

```
FEATURES            Location/Qualifiers
     source         1..229
                    /organism="uncultured Neocallimastigales"
                    /mol_type="genomic DNA"
                    /isolation_source="feces from 18 year old
male horse
```

```
                     'Rio'"
                     /host="horse"
                     /db_xref="taxon:325898"
                     /clone="HorseRio01CAAEG"
                     /environmental_sample
                     /country="USA: Stillwater, OK"
     misc RNA        <1..>229
                     /product="internal transcribed spacer 1"
```

GQ835874

```
FEATURES             Location/Qualifiers
     source          1..229
                     /organism="uncultured Neocallimastigales"
                     /mol_type="genomic DNA"
                     /isolation_source="feces from 18 year old
male horse
                     'Rio'"
                     /host="horse"
                     /db_xref="taxon:325898"
                     /clone="HorseRio04JXXGM"
                     /environmental_sample
                     /country="USA: Stillwater, OK"
     misc RNA        <1..>229
                     /product="internal transcribed spacer 1"
```

GQ836160

```
FEATURES             Location/Qualifiers
     source          1..229
                     /organism="uncultured Neocallimastigales"
                     /mol_type="genomic DNA"
                     /isolation_source="feces from 18 year old male
horse
                     'Rio'"
                     /host="horse"
                     /db_xref="taxon:325898"
                     /clone="HorseRio01BGHO6"
                     /environmental_sample
                     /country="USA: Stillwater, OK"
     misc RNA        <1..>229
                     /product="internal transcribed spacer 1"
```

GQ836227

```
FEATURES             Location/Qualifiers
     source          1..229
                     /organism="uncultured Neocallimastigales"
                     /mol_type="genomic DNA"
                     /isolation_source="feces from 18 year old male
horse
                     'Rio'"
                     /host="horse"
                     /db_xref="taxon:325898"
                     /clone="HorseRio01B8SHC"
                     /environmental_sample
                     /country="USA: Stillwater, OK"
     misc RNA        <1..>229
                     /product="internal transcribed spacer 1"
```

GQ836353

```
FEATURES             Location/Qualifiers
     source          1..229
                     /organism="uncultured Neocallimastigales"
                     /mol_type="genomic DNA"
                     /isolation_source="feces from 18 year old male
horse
                     'Rio'"
                     /host="horse"
                     /db_xref="taxon:325898"
                     /clone="HorseRio04ICT0E"
                     /environmental_sample
                     /country="USA: Stillwater, OK"
     misc RNA        <1..>229
                     /product="internal transcribed spacer 1"
```

GQ836403

```
FEATURES             Location/Qualifiers
     source          1..228
                     /organism="uncultured Neocallimastigales"
                     /mol_type="genomic DNA"
                     /isolation_source="feces  from  18  year  old
male horse
                     'Rio'"
                     /host="horse"
                     /db_xref="taxon:325898"
                     /clone="HorseRio01CD7M9"
                     /environmental_sample
                     /country="USA: Stillwater, OK"
     misc RNA        <1..>228
                     /product="internal transcribed spacer 1"
```

GQ839500

```
FEATURES             Location/Qualifiers
     source          1..228
                     /organism="uncultured Neocallimastigales"
                     /mol_type="genomic DNA"
                     /isolation_source="feces from 18 year old male
horse
                     'Rio'"
                     /host="horse"
                     /db_xref="taxon:325898"
                     /clone="HorseRio01AKG5N"
                     /environmental_sample
                     /country="USA: Stillwater, OK"
     misc RNA        <1..>228
                     /product="internal transcribed spacer 1"
```

GQ842090

```
FEATURES             Location/Qualifiers
     source          1..229
                     /organism="uncultured Neocallimastigales"
                     /mol_type="genomic DNA"
                     /isolation_source="feces from 18 year old male
horse
```

'Rio'"
/host="horse"
/db_xref="taxon:325898"
/clone="HorseRio04H963V"
/environmental_sample
/country="USA: Stillwater, OK"
misc RNA <1..>229
/product="internal transcribed spacer 1"

GQ840318

FEATURES Location/Qualifiers
source 1..229
/organism="uncultured Neocallimastigales"
/mol_type="genomic DNA"
/isolation_source="feces from 18 year old male horse
'Rio'"
/host="horse"
/db_xref="taxon:325898"
/clone="HorseRio04J2DOJ"
/environmental_sample
/country="USA: Stillwater, OK"
misc RNA <1..>229
/product="internal transcribed spacer 1"

GQ843180

FEATURES Location/Qualifiers
source 1..229
/organism="uncultured Neocallimastigales"
/mol_type="genomic DNA"
/isolation_source="feces"
/host="green iguana"
/db_xref="taxon:325898"
/clone="Iguana01A35EC"
/environmental_sample
/country="USA: Department of Zoology, Oklahoma State
University"
misc RNA <1..>229
/product="internal transcribed spacer 1"

GQ850312

```
FEATURES             Location/Qualifiers
     source          1..417
                     /organism="uncultured Cyllamyces"
                     /mol_type="genomic DNA"
                     /isolation_source="cow semiliquid manure"
                     /db_xref="taxon:857191"
                     /clone="89"
                     /environmental_sample
                     /country="Czech Republic"
     rRNA            <1..68
                     /product="18S ribosomal RNA"
     misc RNA        69..300
                     /product="internal transcribed spacer 1"
     rRNA            301..>417
                     /product="5.8S ribosomal RNA"
```

GQ857630

```
FEATURES             Location/Qualifiers
     source          1..600
                     /organism="Neocallimastix sp. AF-CTS-HAN2"
                     /mol_type="genomic DNA"
                     /strain="AF-CTS-HAN2"
                     /isolation_source="hippopotamus feces"
                     /db_xref="taxon:679544"
     misc RNA        <1..>600
                     /note="may contain 18S ribosomal RNA,
internal transcribed
                     spacer 1, 5.8S ribosomal RNA, internal
transcribed spacer
                     2, and 28S ribosomal RNA"
```

GU910843

```
FEATURES             Location/Qualifiers
     source          1..705
                     /organism="uncultured Neocallimastigales"
                     /mol_type="genomic DNA"
                     /isolation_source="dung"
                     /host="bison"
                     /db_xref="taxon:325898"
                     /clone="8WF3ca11"
                     /clone_lib="WCNP"
                     /environmental_sample
                     /country="USA: South Dakota"
                     /collection_date="May-2008"
                     /note="PCR_primers=fwd_name: ITS-1F, rev_name:
ITS-4"
     misc_RNA        <1..>705
                     /note="contains 18S ribosomal RNA, internal
transcribed
                     spacer 1, 5.8S ribosomal RNA, internal
transcribed spacer
                     2, and 28S ribosomal RNA"
```

GU910869

```
FEATURES             Location/Qualifiers
     source          1..704
                     /organism="uncultured Neocallimastigales"
                     /mol_type="genomic DNA"
                     /isolation_source="dung"
                     /host="bison"
                     /db_xref="taxon:325898"
                     /clone="8WF3cd02"
                     /clone_lib="WCNP"
                     /environmental_sample
                     /country="USA: South Dakota"
                     /collection_date="May-2008"
                     /note="PCR_primers=fwd_name: ITS-1F, rev_name:
ITS-4"
     misc_RNA        <1..>704
```

/note="contains 18S ribosomal RNA, internal
transcribed
spacer 1, 5.8S ribosomal RNA, internal
transcribed spacer
2, and 28S ribosomal RNA"

GU910896

FEATURES Location/Qualifiers
source 1..707
/organism="uncultured Neocallimastigales"
/mol_type="genomic DNA"
/isolation_source="dung"
/host="bison"
/db_xref="taxon:325898"
/clone="8WF3cf12"
/clone_lib="WCNP"
/environmental_sample
/country="USA: South Dakota"
/collection_date="May-2008"
/note="PCR_primers=fwd_name: ITS-1F, rev_name:
ITS-4"
misc_RNA <1..>707
/note="contains 18S ribosomal RNA, internal
transcribed
spacer 1, 5.8S ribosomal RNA, internal
transcribed spacer
2, and 28S ribosomal RNA"

GU911073

FEATURES Location/Qualifiers
source 1..717
/organism="uncultured Neocallimastigales"
/mol_type="genomic DNA"
/isolation_source="dung"
/host="bison"
/db_xref="taxon:325898"
/clone="8WF6ch02"
/clone_lib="WCNP"
/environmental_sample

/country="USA: South Dakota"
/collection_date="May-2008"
/note="PCR_primers=fwd_name: ITS-1F,
rev_name: ITS-4"
misc RNA <1..>717
/note="contains internal transcribed spacer
1, 5.8S
ribosomal RNA, internal transcribed spacer 2,
and 28S
ribosomal RNA"

JN560945

FEATURES Location/Qualifiers
source 1..810
/organism="Anaeromyces sp. NRFI-4"
/mol_type="genomic DNA"
/strain="NRFI-4"
/isolation_source="rumen liquor"
/host="cattle; breed: Karan Fries"
/db_xref="taxon:1078493"
/country="India"
misc_RNA <1..>810
/note="contains 18S ribosomal RNA, internal
transcribed
spacer 1, 5.8S ribosomal RNA, internal transcribed
spacer
2, and 28S ribosomal RNA"

JQ782544

FEATURES Location/Qualifiers
source 1..1475
/organism="Neocallimastix sp. NYF3"
/mol_type="genomic DNA"
/strain="NYF3"
/db_xref="taxon:1253876"
rRNA <1..30
/product="18S ribosomal RNA"
misc RNA 31..285
/product="internal transcribed spacer 1"
rRNA 286..444

```
                    /product="5.8S ribosomal RNA"
     misc RNA       445..655
                    /product="internal transcribed spacer 2"
     rRNA           656..>1475
                      /product="28S ribosomal RNA"
```

JQ782546

```
FEATURES            Location/Qualifiers
     source         1..1490
                    /organism="Neocallimastix sp. NYR1"
                    /mol_type="genomic DNA"
                    /strain="NYR1"
                    /db_xref="taxon:1253878"
     rRNA           <1..30
                    /product="18S ribosomal RNA"
     misc RNA       31..296
                    /product="internal transcribed spacer 1"
     rRNA           297..455
                    /product="5.8S ribosomal RNA"
     misc RNA       456..670
                    /product="internal transcribed spacer 2"
     rRNA           671..>1490
                    /product="28S ribosomal RNA"
```

JQ782549

```
FEATURES             Location/Qualifiers
     source          1..1476
                     /organism="Neocallimastix sp. NYR4"
                     /mol_type="genomic DNA"
                     /strain="NYR4"
                     /db_xref="taxon:1253881"
     rRNA            <1..30
                     /product="18S ribosomal RNA"
     misc RNA        31..284
                     /product="internal transcribed spacer 1"
     rRNA            285..443
                     /product="5.8S ribosomal RNA"
     misc RNA        444..655
                     /product="internal transcribed spacer 2"
     rRNA            656..>1476
                     /product="28S ribosomal RNA"
```