**UNIVERSITÀ DEGLI STUDI DI SASSARI**

***CORSO DI DOTTORATO DI RICERCA in***

**SCIENZE VETERINARIE**

*INDIRIZZO*: **Produzione, Qualità e Sicurezza Alimentare (XXIX CICLO)**

# Methanogenic community structure in the digestive tract of Equidae

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**Tesi di dottorato della**

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## Ringraziamenti

Al termine di questi tre anni di dottorato sento il dovere e il piacere di ringraziare tutte le persone che, con i loro consigli e il loro affetto, mi hanno accompagnato e sostenuto in questo percorso e senza le quali questo lavoro di tesi non sarebbe stato possibile.

Innanzitutto voglio ringraziare il Prof. Giuseppe Moniello che con la sua grande disponibilità ed infinita pazienza ha saputo guidarmi e indirizzarmi in questo percorso, dandomi sempre preziosi suggerimenti in tutte le fasi del mio lavoro.

Ringrazio di cuore, per tutto l'aiuto offertomi, sia da un punto di vista scientifico che morale, la Dott.ssa Kateřina Fliegerová, dimostratasi persona unica e speciale in tutto il periodo che ho trascorso nella sua bellissima Praga e per tutti i suoi insegnamenti nell'ambito della ricerca, dimostrandosi una guida ed un punto di riferimento per me.

Il mio ringraziamento va anche a tutto il gruppo dell'"Institute of Animal Physiology and Genetic" per l'ospitalità offertami durante la mia permanenza a Praga.

Un ringraziamento particolare va anche alla Prof.ssa Serena Calabrò e al Dott. Jakub Mrázek per i preziosi suggerimenti dati in qualità di revisori esterni permettendo di migliorare la presente tesi.

Un sincero grazie a tutti i miei amici italiani ed internazionali sui quali ho sempre potuto fare affidamento nei momenti di gioia e di sconforto del percorso di Dottorato.

Ringrazio, infine, immensamente la mia famiglia per non avermi fatto mai mancare il proprio sostegno morale, sostenendomi in ogni mia scelta e spronandomi sempre ad andare avanti per la mia strada.

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# Abstract

The aim of the present study was to assess the diversity of the methanogen community in the different segments of the horse and donkey hindgut (caecum, right ventral colon, left ventral colon, left dorsal colon, right dorsal colon and rectum).

In the present study the diversity of methanogens was investigated by constructing 16S rDNA gene libraries, using specific archaeal primers, Met86F and Met1340R. The microbial genomic DNA was isolated from two adult animals, a male horse and a female donkey. The amplified product was cloned into a suitable vector and the positive clones were selected, reamplified and the resulting PCR products were further subjected to RFLP (Restriction Fragment Length Polymorphism) analysis by using the restrictases MluI and MboI. A total of 680 clones generated from twelve archaeal 16S rDNA gene cloning libraries of different segments of horse (345 positive clones) and donkey (335 positive clones) hindgut were examined and the analysis revealed 23 ribotypes as defined by RFLP analysis. The diversity of the hindgut methanogen population was higher in the horse compared to the donkey. The phylogenetic analysis of the archaeal 16S rRNA gene sequences of each species revealed interesting differences among the different parts of the digestive tract and considerable diversity between these two species. In the horse the phylogenetic analysis showed that the clones were classified into 4 clades: Methanobacteriales, Methanomicrobiales, Methanomassiliicoccales and Methanosarcinales. The clones of donkey were classified in 3 clades: Methanobacteriales, Methanomassiliicoccales and Methanomicrobiales. The abundance and the distribution of methanogens differed greatly in the two animals, while the methanogen community structure was quite similar, with the dominance of Methanomicrobiales and Methanobacteriales. This finding indicates that hydrogenotrophic methanogenesis is the major methane production pathway in the hindgut of both the species. The absence of Methanobrevibacter species and the dominance of Methanocorpusculum species in the caecum of both the animals reflect the specific metabolic processes in this part of digestive tract and indicate the possible differences in the metabolic activities among the hindgut segments of equidae.

# Introduction

The digestive tract of mammals, including humans, is inhabited by a complex ecosystem, the intestinal microbiome. Animals can be divided, according to their digestive physiology and the gastrointestinal microorganisms can be examined according to their gut location (Stevens and Hume, 1998): foregut (before gastric stomach), midgut (small intestine) and hindgut (large intestine). The microbial composition of the gut microbiome varies across the digestive tract. In the stomach and small intestine, relatively few species of bacteria are generally present (Guarner and Malagelada, 2003). The colon, in contrast, contains a densely-populated microbial ecosystem with up to $10^{12}$ cells per gram of intestinal content (Guarner and Malagelada, 2003). Over 99% of the bacteria in the gut are anaerobes. Microorganisms in the gut assist mainly in the digestion of feedstuffs, supply the host by energy, producing end fermentation products, help to protect the animal from infections and some microbes even synthesize and provide essential nutrients to their animal host.

The complex anaerobic microbial communities consist of many species from divergent groups such as bacteria, protozoa, fungi and archaea (Wolin, 1979). The microbiome of the gastrointestinal tract in animals has been studied intensively. These studies have demonstrated that bacteria are the most numerous microbes present in the gastrointestinal tract. The composition of the bacterial populations varies from animal to animal, even within a species (Hao and Lee, 2004).

Through the combined activity of these microorganisms, macromolecules such as polysaccharides, proteins and lipids are metabolized into smaller organic molecules such as volatile fatty acids, which are absorbed by the host to provide energy. However, other products of microbial anaerobic respiration, including carbon dioxide and methane, are not assimilated and are released into the environment. The Methane emissions from livestock are problematic for two reasons. First, the enteric production of methane has a negative impact on animal productivity, resulting in lost energy, ranging from 2% to 12% of the animal's gross energy intake (Johnson and Johnson, 1995). Second, once released into the environment, methane acts as a potent greenhouse gas with 25 times the global warming potential of carbon dioxide.

Methane is produced by obligate anaerobic microorganism called methanogens, a diverse group of obligate anaerobic archaea that share methane synthesis as the end product of their anaerobic respiration (Liu and Whitman, 2008). Archaea form a large and diverse domain, both phylogenetically and ecologically, and have been identified in a wide variety of environments

like marine and freshwater sediments, soils, hot springs, sewage sludge and the digestive tracts of animals and humans.

So far, methanogens have been isolated from various animals. The principal source of methane is from ruminant animals, which is formed in the rumen through a process called enteric fermentation (Ellis *et al*., 2007). In the recent years, interests in methanogenic bacteria from ruminating animals have been extended (Hook *et al*., 2010). A number of experimental approaches have been carried out to reach the knowledge of the methanogens population in the rumen of animals. It has been estimated that a domesticated ruminant, such as cattle, sheep and goats produces 86 million metric tons of methane per year. Approximately 18.9 Tg are from dairy cattle, 55.9 Tg are from beef cattle, and 9.5 Tg are from sheep and goats (McMichael *et al*., 2007). Non-ruminants also contribute to methane emissions through enteric fermentation in the large intestine, but in much smaller quantities than ruminants (Wang and Huang, 2005). However, methane production by large herbivorous monogastric animals such as horses, mules and donkeys is substantial, up to 80 l per animal per day.

To date, the studies of methanogenic bacteria present in monogastric animals, like members of the Equidae family, are still scarce. Compared to the rumen microbiota, the equine hindgut microbiota has received little attention. Only limited information is available on the methanogen population in the hindgut of horses (Lwin and Matsui, 2013, Fernandes *et al*., 2014), while almost nothing is known about the methanogenic community structure in the donkey. Information about the methanogen density and diversity in the hindgut of horse and donkey are important for understanding the microbial ecosystem of their hindgut. In the present study, the diversity of methanogens was explored along the different tracts of the horse and donkey hindgut using molecular approaches based on the 16S rRNA gene.

# 1. THE DIGESTIVE SYSTEM AND THE BACTERIAL COMMUNITY OF THE EQUIDS FAMILY

## 1.1 Equine family

*Equidae* is a family within the order Perissodactyla (the odd-toed ungulates), and it has only one extant genus, *Equus*, described for the first time by Carl Linnaeus in 1758. The genus *Equus* includes seven species: *E. africanus* (African Wild Asses), *E. ferus* (Wild Horse), *E. gravyi* (Grevy's Zebras), *E. hemionus* (Onagers), *E. kiang* (Kiangs), *E. quagga* (Plains Zebras), *E. zebra* (Mountain Zebras), and numerous subspecies. Species and subspecies of Equus can interbreed and form hybrids. There are approximately 35 other genera within the family *Equidae*, which are all extinct (Groves and Willoughby, 1981).

All members of the Equidae family share the basic physical qualities but differ somewhat in conformation. They are large animals, ranging in body size from around 200 to 500 kg. The general form of equids presents long and slender legs and a very developed large third toe encased in a hoof. The facial part of the skull is greatly elongated, with long and sturdy necks. A characteristic bristly mane is found on the nape of the neck, and the tail has a long tassle that ends either in a tuft, or entirely covered in flowing hair. The equids evolved so as to adapt to fast running in open spaces and eating coarse vegetation. They are adapted to generally open terrain, from plains and savannas, to mountains or deserts.

The pinnae ("ears") of equids are mobile, enabling them to easily localize the origin of sounds. Their eyes are set back far on the head, giving them a wide angle of view, without entirely losing binocular vision (Moehlman, 2004).

Equines are primarily grazers; they utilize plant fiber (largely cellulose) that comes from grass and hay but can eat fruits and leaves when needed. They have specialized digestion, they are hindgut fermenters and are not like cows and other ruminants which use multiple stomach compartments and re-chew their food (Franzen, 2010). Unlike ruminants, with their complex stomachs, equids have only one stomach and break down cellulose in the "hindgut" or large colon. Their dentition is almost complete, they have large molars for grinding grasses with high content of fibers and large incisors highly crowned (Hansen, 1976; Bennett and Hoffmann, 1999).

### 1.1.1 Horse

The horse (*Equus ferus caballus*) is one of extant subspecies of genus Equus (Bennett and Hoffmann, 1999). At present, the domesticated and wild horses are considered a single species, with the valid scientific name for the horse species being *Equus ferus* (Bennett and Hoffmann, 1999).

Horses are large herbivorous mammals that have long slender legs ending in a single toe covered by a horny hoof. A horse's mane and tail is generally long and thick. A horse's coat is much smoother than the coarse hair of the other equines. Horses are adaptable and occupy a wide variety of habitats under domestication. Preferred habitats are cool, temperate grasslands, steppes and savannahs, but they also occupy semidesert, swamps, marshes and woodlands (Bennett and Hoffmann, 1999). They are a social animal that prefer to live in large herds. While most are domesticated and live with humans, others remain wild. Horses are built for speed and running long distances, they are the only other mammal besides humans that can produce large amounts of sweat for thermoregulation. The size and body structure will vary depending on the breed and the use to which this is intended. Horses' anatomy enables them to make use of speed to escape predators and they have a well-developed sense of balance and a strong fight-or-flight response (Bennett and Hoffmann, 1999).

Horses primarily graze grasses. Domestic horses are often fed with various amounts of grains, including oats, barley, corn, wheat, flax, soybeans, alfalfa, clover, hay and grass. In addition to this diet, they may also be provided by vitamin and mineral supplements. Feral horses eat a similar diet, which varies depending on location and season (Hansen, 1976; Bennett and Hoffmann, 1999).

### 1.1.2 Donkey

The donkey (*Equus africanus asinus*) is a domesticated member of the family Equidae (Beja-Pereira, 2004). The wild ancestor of the donkey is the African wild ass, *E. africanus* (Rossel *et al*., 2008).

Donkeys differ from horses in conformation. Donkeys are often, but not always, smaller than horses. The most noticeable difference is of course the ears. Donkey's ears are much longer in proportion to their size than a horse's. The mane and tail in the donkey are coarse. The mane is stiff and upright and the tail is covered with short body hair for most of the length. Hoof shape varies as well, donkey hooves are smaller and rounder, with more upright pasterns. Compared to

horses, all donkeys possess specialized teeth and long limbs. The adaptation of long limbs and narrow hooves allow these animals to move swiftly and easily through rocks.

Donkeys are adapted to live in mountainous and desert areas. Unlike horses, wild donkeys in dry areas are solitary and do not form harems. In arid and semi-arid areas, donkeys eat a wide variety of feeds. The donkeys graze, eat forbs and shrubs and the bark of trees. They normally should be allowed to graze for six to seven hours a day on free range. A donkey, like the horse, is a monogastric herbivore eating roughages and utilizing cellulose and hemicellulose efficiently. They are extremely efficient at digesting fibrous, poor quality plant material and have evolved as browsers (eating woody shrubs and trees) as well as grazers (Smith and Wood, 2008).

The donkey's superior digestive ability makes them ideally suited to hard work in areas where food is poor of quality or is limited. Even if there is no marked structural difference between the gastro-intestinal tract of a donkey and that of a horse, the digestion of the donkey is more efficient (Taylor, 1997). It needs less food than a horse or pony of comparable height and weight (Smith and Wood, 2008), approximately 1.5 % of body weight per day in dry matter, compared to the 2–2.5 % consumption rate possible for a horse (Smith and Pearson, 2005). Donkeys are also less prone to colic. The reasons for this difference are not fully understood; the donkey may have different intestinal microbiome compared to the horse, or a longer gut retention time (Jane, 1997).

## 1.2 Equine digestive anatomy and physiology

Horses and other equids evolved like hindgut fermenting grazing animal and, being a nonruminant herbivore, bring together the advantages of enzymatic digestion along the small intestine, (which is that of monogastric), and a long and intense microbial fermentation in the large intestine (similar to ruminants). The enzymatic digestion allows to get the best performance from carbohydrates, lipids, proteins, and vitamins, while the microbial fermentation offers the possibility to take advantage of fibrous foods and a recycling nitrogen (Santos *et al.*, 2011). The digestive system is composed of the alimentary canal and its accessory organ. The alimentary canal is a tube, which extend from the mouth to the anus and can be divided in two sections (figure 1.2- 1). The first section, the foregut, consisting of the mouth, esophagus, stomach and small intestine, and the second section, the hindgut, consisting

of the caecum, large and small colon and rectum. Glands and accessory organs, like liver and pancreas are situated outside the GI tract and are involved also in the digestion processes. The gross anatomy of the intestine of the donkey is similar to the domestic horse in general, in spite of some differences in dimensions (Jerbi *et al.* 2014).

Digestion is the process of preparation of food for absorption of nutrients from the alimentary canal into the bloodstream and elimination of the waste residue from the body through a series of combined effects of mechanical, secretory, chemical and microbiological factors.

Each part of the equine digestive tract performs an important function and the next sections briefly describe the anatomic features and functions of the different segments of the GI tract.

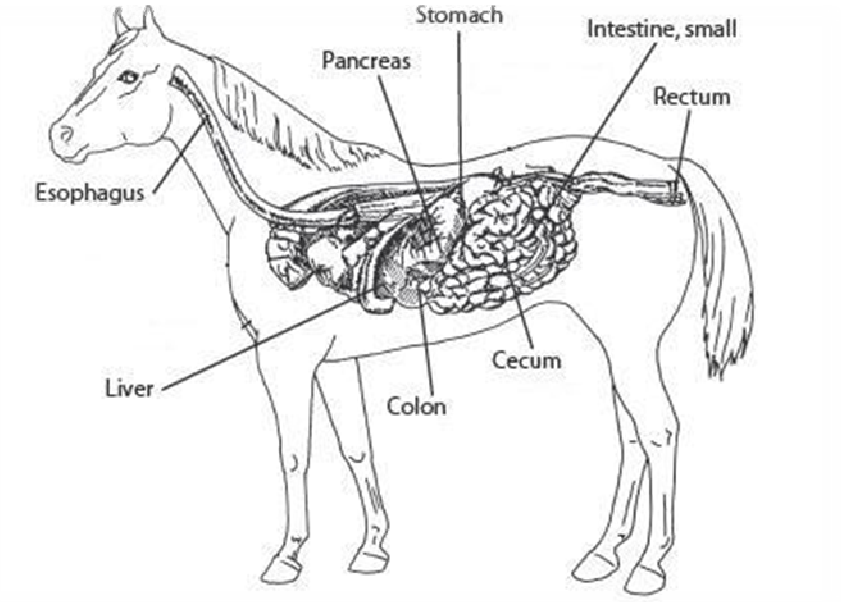


**Figure 1.2-1. Anatomy of equine digestive tract**

### 1.2.1 Mouth

The digestive system starts with the mouth, which contains different structures like lips, tongue, teeth and the associated salivary glands, which aid in the prehension (first act of digestion) and the mechanical disintegration of food into finely divided particles, which provide a greater surface area for the action of digestive juice.

The lips, which are strong, mobile, and sensitive, are used to select grasses, hay, and grains and to funnel water into the mouth. The upper and lower incisors shear forages close to the ground and then the tongue helps move ingested material to the cheek teeth for grinding (Frape, 2010). Saliva (about 10 to 12 L per day) is secreted only in response to the presence of the food in the mouth and to the chewing action and it helps to wet and lubricate the chewed feed or digesta to moving through the digestive tract (Al Jassim and Andrews, 2009). Chewing the feed into smaller particles is important because it allows for nutrients like glucose, calcium, and vitamin A to be readily absorbed in subsequent section of the digestive tract (Frape, 2010).

### 1.2.2 Esophagus

The esophagus is a muscular tube about 1.5m in length and its mainly function is to move the chewed feed, known as digesta, from the mouth to the stomach (Al Jassim and Andrews, 2009). At the end of the esophagus, there is a tight muscular valve nicknamed the one-way cardiac sphincter, which remains closed except during swallowing, because its function is to ensure movement of digesta into the cardiac region of the stomach, but not back into the esophagus. Because this mechanism does not allow to horses to regurgitate and expel what has been eaten, horses should be fed good quality feed and forage (Al Jassim and Andrews, 2009).

### 1.2.3 Stomach

The stomach, in relation to the size of the animal, is relatively small. It represents only 10% of the GI tract with a net capacity of 7.5 to 15L, depending on feed type. The equine stomach has two areas; the non-glandular or squamous area, where food enters from the esophagus, and the glandular, where it meets gastric juices for digestion (Al Jassim and Andrews, 2009). Gastric juice contains hydrochloric acid (HCl) that lowers the pH of the stomach's contents, and two enzymes, pepsin and gastric lipase. Proteins are digested in the stomach by pepsin. Gastric lipase helps start the digestion of fats into fatty acids and glycerol. Not all the juices, that help the digestion, originate in the stomach; some of the juices come from the pancreas. There is actually a constant secretion of pancreatic juice, which helps with the breakdown of the cellulose material. It takes on average 2-4 hours for digesta that has entered the stomach to move into the small intestine (Lewis, 1996).

### 1.2.4 Small Intestine

The small intestine is approximately 15 to 21 m long and isthe major site where the enzymatic digestion continues and where important absorption of nutrients occurs. It consists of three sections: the duodenum, the jejunum and the ileum (Frape, 2010). The main function of the small intestine is to complete digestion of simple carbohydrates (sugar and starch), lipids (fats and oils), amino acids, vitamins and minerals from the stomach.

The pancreas and liver assist the small intestine with digestion. The pancreas secretes a mixture containing enzymes (amylolytic, proteolytic and lipolytic enzymes), which aid in breaking down protein into amino acids and complex carbohydrates into simple sugars. Pancreatic juice is secreted continuously in the horse and ensures also helps raise the pH of the digesta for optimal microbial fermentation or digestion of feed by gut microbes. The liver produces a compound known as bile, which emulsifies fat and hence improves enzymatic digestion and assists with fat absorption by the small intestine. However, bile is continuously secreted by the liver because the horse does not have a gall bladder (Al Jassim and Andrews, 2009).

Digestion and absorption of carbohydrates, proteins and fat is more efficient in the duodenum than in the other two segments of the small intestine (Jackson, 1998).

The food in the small intestine is pushed with muscular contractions and it takes from 45 minutes to two hours to pass through the small intestine depending on meal size, amount of forage and size of horse (Frape, 2010).

### 1.2.5 Hindgut (Large intestine)

The material that is not or cannot be digested in the small intestine passes into the large intestine. The equine large intestine (hindgut) is approximately 8m long and is divided into the caecum, large colon, small colon, rectum, and terminates at the anus (figure 1.2-2). The hindgut is the site where the complex insoluble carbohydrates, cellulose and hemicelluloses, are digested by fermentation by resident microbial population into simpler compounds, which can be absorbed through the gut wall. Microbial fermentation in the hindgut results in the production of volatile fatty acids (VFAs), such as acetate, propionate and butyrate, which are an important nutrient source for the horse which represent about 60-70 % of a body energy (St-Pierre *et al.*, 2013). The pH of the caecum and colon is approximately 6.0 and forms the ideal condition for anaerobic bacteria, fungi and protozoa to degrade (hemi)celluloses and pectins (Bonhomme-Florentin, 1988). The hindgut acts also as a reservoir of water and electrolytes, which is

essential for working horses. The microbial population is highly susceptible to dietary changes often resulting in digestive upsets such as diarrhea, colitis, laminitis or colic. The numbers and species of microbes within the horse's hindgut vary depending upon factors such as host species, diet and geographical location. The microbiota of an herbivore typically consists of bacteria, archaea, protozoa and fungi, with bacteria representing the dominant part (Hobson, 1997).

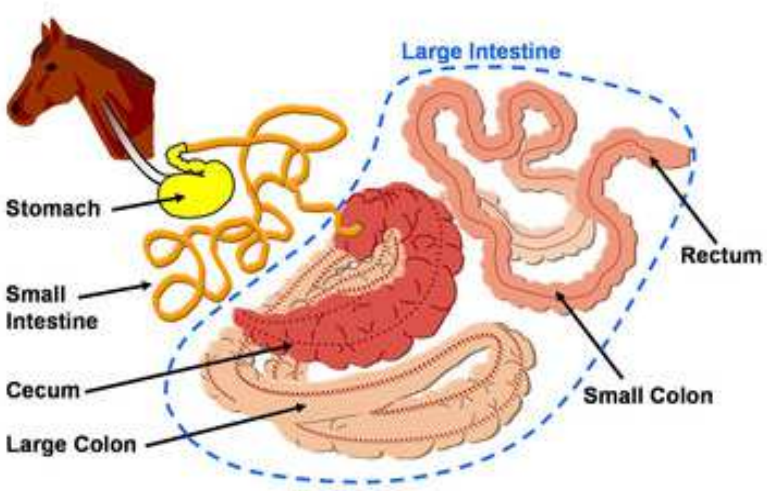


**Figure 1.2-2. Equine large intestine.**

### *1.2.5.1 Caecum*

The large intestine in the horse opens directly into the caecum through a muscular valve, called ileo-cecal junction (Al Jassim and Andrews, 2009). The caecum is a large blind sac, approximately 1.2m long, where the digesta appears after 30-45 min after leaving the stomach and approximately 3h after feed consumption (Van Weyenberg *et al.,* 2006). Here the undigested nutrients are fermented in a process similar to that which occurs in the forestomach of ruminants. In the caecum, microbes ferment and break down the fiber that horse consumes to get important nutrients such as volatile fatty acids and some amino acids that would otherwise be wasted. Food remains in the caecum for up to seven hours, ensuring to the microbes to have adequate time to digest the feed material. Energy producing volatile fatty acids, amino acids, and B vitamins are a result of this fermentation process that are then reabsorbed in the caecum.

The microbial population in the caecum becomes specific for the type of food that the horse normally consumes.

### *1.2.5.2 Large Colon*

In the horse, the large colon is folded, so it forms a double loop consisting of 4 segments: Right Ventral Colon (RVC), Left Vental Colon (LVC), Left Dorsal Colon (LDC), Right Dorsal Colon (RDC). From the caecum digesta flows out into the right ventral colon, which has a retention time of 3h, and here the largest proportion of fibre fermentation takes place. The proportion of cellulose in the digesta decreases as it reaches the ventral and then dorsal colon (De Fombelle *et al.,* 2003). The ventral colon (RVC and LVC) is voluminous with a diameter up to 25 to 30 cm and about 2 to 4 m long, while the transition between the LVC and the left dorsal colon (LDC) is narrow, and this anatomic morphology delays the transport of large particles from the ventral to the dorsal colon, increasing their retention time and fermentability, and only liquid and fine particles move on to the LDC and RDC (Van Weyenberg *et al.,* 2006). In this section of large intestine the microbial digestion (fermentation) continues, and most of the nutrients made through microbial digestion are absorbed here as well as B group vitamins produced by the bacteria and some trace minerals and phosphorous (Schryver *et al.*, 1972; Frape, 2010).

### *1.2.5.3 Small Colon and Rectum*

The small colon is approximately 4 m long and the main function is to absorb water which helps to keep the horse hydrated and to form fecal balls. Faeces is the waste matter of digestion and contains water, indigestible and undigested food residues, cells sloughed off the intestinal wall, and remains of digestive secretions. The rectum is approximately 30 cm long and, being the terminal part of the digestive tract, the main function is to excrete faeces (Al Jassim and Andrews, 2009).

## 1.3 The microbiome of the equine

The equine large intestine (caecum and colon) is an immensely enlarged fermentative chamber which contains an abundant and complex community of microorganisms. It is estimated that 30% (Kern *et al.,* 1973) to 80% (Kern *et al.,* 1974) of the caecum and colon microbial population is strictly anaerobic.

This community includes bacteria, fungi, protozoa and archaea and plays an important role in the health, growth, development and the performance of the animal. The microbial digestive activity within the caecum and colon enables the horse to obtain nutrients from the ingested forage. The digesta rich in cellulose reaches the caecum after a minimal degradation in the foregut parts; any starch that has bypassed enzymatic breakdown in the small intestine will also appear in the caecum to be fermented.

In the hindgut, the neutral pH, the availability of fibre and the long residence time of digesta support a diverse microbial population and each type is efficient to digest certain nutritional components (Daly *et al.*, 2001).

Many microbial species are involved in fermenting complex polysaccharides, changing them into a form that is digestible by the horse. The main products of microbial fermentation of complex polysaccharides are volatile fatty acids (VFAs). The VFAs diffuse into the blood and represent the main source of energy for the horse. The main VFAs produced are acetate, propionate and butyrate with isobutyrate, isovalerate and valerate produced in smaller amounts (Mackie and Wilkins, 1988). Mackie and Wilkins (1988) reported the molar proportions of acetate, propionate and butyrate in the hindgut 85:10:3, respectively.

Under normal feeding conditions, the microbial population maintains, instead, a balance with its host, keeping the integrity of the ecosystem, contributing to the prevention of disorders and forming a barrier against pathogens (Jandhyala *et al.*, 2015). The microbial population is highly susceptible to dietary changes, often resulting in digestive upsets, such as diarrhea, colitis, laminitis or colic. The numbers and species of microbes within the equids's hindgut vary depending upon factors such as ingredients and any sudden changes to the diet. A change from a normal fibre-rich diet to a starch-rich diet will modify the microbial population and the end-products of microbial fermentation in the hindgut. The microbial population and activity within the different parts of the digestive tract is a factor of both the anatomy and physiology of the gut and the substrate supplies that reach that part.

Despite the importance of microbes in supplying energy, little is still known about the overall composition of the microbial community (microbiome) in the equine hindgut. Information relating to the quantification, characterisation and metabolic activity of the microbial population in the horse GIT is limited (Costa and Weese, 2012) and our knowledge of the microbiome composition has increased in very different ways, depending on the community (Julliand and Grimm, 2016). For each microbial community, we will present its quantification, its diversity, and its implication in plant fiber degradation.

### 1.3.1 Bacteria

In terms of quantity, the bacterial community represents the majority of the hindgut microbiota. As early as 1911, Choukevitch classified the equine hindgut bacteria under their morphology and wall composition using microscopic examination and Gram staining. Today, most of the studies on equine hindgut bacteria is based on the molecular techniques, such as PCR, fingerprint techniques and, lately, high-throughput next-generation sequencing, which enormously enlarged our knowledge and identified some novel uncultured genera (Julliand and Grimm, 2016).

There is a great diversity regarding the bacterial activity in the equine hindgut and the concentration of several functional groups of bacteria has been reported in it. Bacteria can be classified into glycolytic, starch-utilizing bacteria and lactate-utilizing bacteria, which present the same concentration along the all large intestine (about $10^8$ cells/mL); into cellulolytic ($10^4$ to $10^7$ and $10^4$ to $10^8$ cells/mL, respectively), hemicellulolytic ($10^7$ to $10^8$ and $10^6$ to $10^8$ cells/mL, respectively), pectinolytic ($10^7$ to $10^8$ and $10^6$ to $10^7$ cells/mL, respectively) and proteolytic ($10^8$ and $10^6$ to $10^8$ cells/mL, respectively) as described by Muhonen *et al.* (2010) and Saudet-Bourgeteau and Julliand (2012).

Within the content of the caecum and the colon, *Firmicutes* and *Bacteroidetes* constitute the most abundant two phyla (Dougal *et al.,* 2013; Moreau *et al.,* 2014; Hansen *et al.,* 2015). 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 the order Bacteroidales, family *Prevotellaceae*.

The cecal cellulolytic microbiome is mainly composed of specific strains of ruminococcus, in particular the strains of *Ruminococcus flavefaciens*, *Ruminococcus albus* and *Fibrobacter succinogenes* are dominant (Jullian *et al.,* 1999).

### 1.3.2 Protozoa

Protozoa are a group of unicellular eukaryotic organisms and are divided in five phyla: *Mastigophora, Sarcodina, Ciliophora, Opalinida* and *Sporozoa*. The Protozoa found in the different compartment of the hindgut are members of the *Ciliophora* phylum, with about 50 species belonging to almost 30 genera of ciliates. The genera *Blepharocorys* and *Cycloposthium* appear to be the most common (Julliand and Grimm, 2016).

The ciliate protozoa are capable to ferment carbohydrates by the cellulolytic activity, to degrade pectin substance by pectin lyase and pectinesterase enzymes (Bohomme-Florentin, 1988).

For many decades, protozoa were considered to be likely contributors to plant fiber breakdown. However, some studies conducted by Moore and Dehority (1993) led to the conclusion that the removal of protozoa from the hindgut of the equine (defaunation) resulted in only a slight decrease in the overall digestibility and had no effect on the cellulose digestion, indicating that protozoa would not play an essential role in the degradation of plant fiber. Therefore, the role of protozoa in the hindgut of horse is not well understood.

### 1.3.3 Fungi

In 1961, Batista *et al.* reported the presence of fungi in the equine hindgut, which, like other fungi living in the digestive ecosystems, have an original and specific feature, they are strictly anaerobic. This imposes severe constraints for culture-based approaches and has driven research toward the application of molecular techniques (Julliand and Grimm, 2016). Recently, fungi were quantified in the hindgut of horse using specific primers targeting a conserved region of the 5.8S rRNA gene (Dougal *et al*., 2013). Despite their low concentration in the digestive tract, anaerobic fungi, in association with bacteria and protozoa, are able to play an important role in fiber degradation.

Furthermore it is estimated that the digestive tract of Equide is occupied by completely unknown anaerobic fungi, the uncultured *Neocallimastigales* represent the majority of the anaerobic fungi (81%) (Mura, 2013) and the known genera of anaerobic fungi were found only

in limited number: *Cyllamyces* 4-12%, *Neocallimastix*2%, *Piromyces* 0.3%, *Anaeromyces* 0.1-0.3% (Liggenstoffer *et al.,* 2010).

### 1.3.4 Archaea

The Archaea of the equine digestive tract are strictly anaerobic methanogens, which use $H_2$ and $CO_2$ to produce methane ($CH_4$) (Jensen, 1996). Archaea were identified for first time in the horse caecum in 1996 by Morvan *et al.* and in the same year Jensen (1996) identified species of *Methanobrevibacter*as the predominant methanogen in the horse faeces. The abundance of methanogenic archaea relative to total bacteria were measured by quantitative PCR (Dougal *et al*., 2013) and was found to be greater in the right dorsal colon than in the caecum.

Recent study conducted by Lwin and Matsui (2014) and Fernandes *et al.* (2014) on the comparison of the methanogen diversity in horse and pony have reported that the phylum *Methanomicrobiales* was the most abundant group in their hindgut and most of the clones obtained in this study were unidentified methanogens, showing that the ecosystem is still an unexplored environment. In fact, to date only limited information is available on the methanogen population in the hindgut of equids.

# 2. METHANE-PRODUCING ARCHAEA: METHANOGENS

## 2.1 Methanogenic archaea

Methanogenic archaea, also called Methanogens or methane-producing Archaea, are a group of anaerobic prokaryotes, which share a unique energy metabolism resulting in the production of methane. All methanogens belong phylogenetically to the domain Archaea, the third domain of life, distinct from the other two domains – Bacteria and Eukarya (Figure 2.1-1). Archaea domain was proposed in 1977 by C. R. Woese on the basis of 16S rRNA sequence (Woese *et al.* 1990).

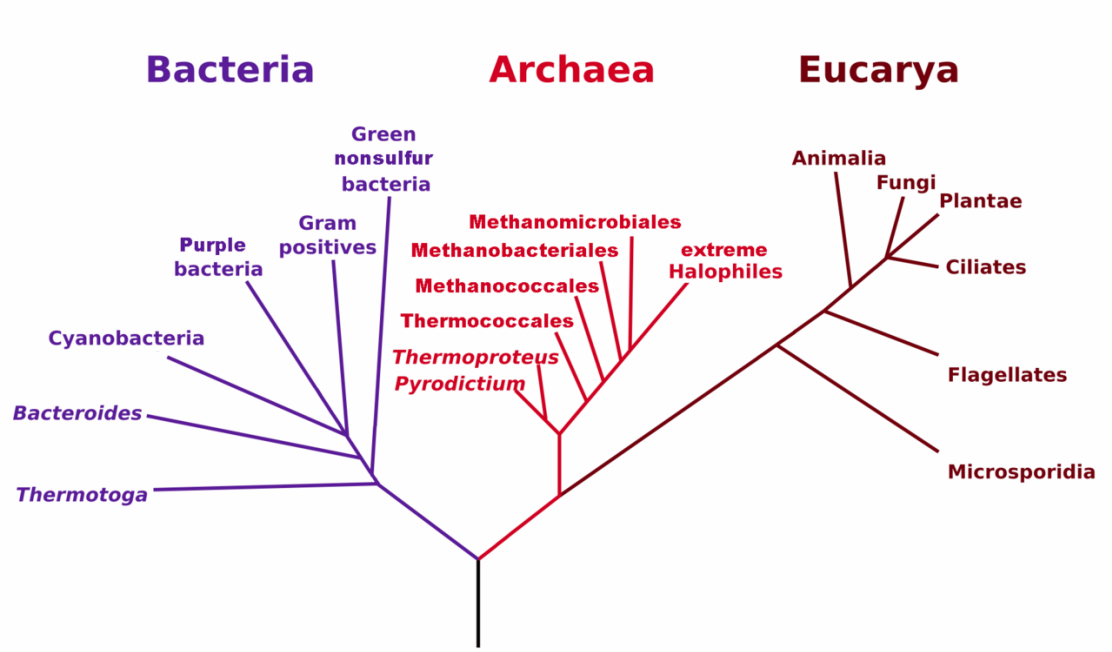


**Figure 2.1-1:Phylogenetic tree of life**

Although Archaea form an independent group of the prokaryotes, they share traits with both Bacteria and Eukaryotes. Like Bacteria, they are unicellular microorganisms that lack a nuclear membrane and intracellular compartmentalization (Sowers, 2009). However, the cell membranes and the surface layers in Archaea are different from those of Bacteria. The main feature distinguishing Bacteria from Archaea is the nature of their cell envelope. In contrast to Bacteria, the archaeal membrane is composed of isoprenoids (and not fatty acids) ethers (and

not esters) linked to glycerol or carbohydrates (Grant *et al*., 1985) and the archaeal envelope does not contain peptidoglycan murein, resulting therefore in insensitivity to the antibiotics which inhibit the synthesis of the cell wall in Bacteria, such as penicillin, cycloserine and valynomycin (Hilpert *et al.,* 1981). Instead, the archaeal cell envelope is composed of a single membrane surrounded by a proteinaceous layer called the S-layer, or it can also contain pseudomurein, depending on the order. In contrast, several molecular features of the Archaea have homology to Eukarya; these features include histone-like DNA proteins, a large multicomponent RNA polymerase and Eukarya-like transcription initiation (Sowers, 2009). The following table (Table 2.1-1) compares some major characteristics of the three domains, to illustrate their similarities and differences.

**Table 2.1-1. Comparison of the major characteristics of three domains.**

| Features | Archaea | Bacteria | Eukarya |
|---|---|---|---|
| **Cell Membrane** | Ether-linked lipids, S-layer | Ester-linked lipids, peptidoglycan | Ester-linked lipids |
| **Gene structure** | Circular chromosome, Similar translation and transcription to Eukarya | Circular chromosomes, unique translation and transcription | Multiple, linear chromosomes, similar translation and transcription to Archaea |
| **Internal Cell Structure** | No membrane-bound organelles or nucleus | No membrane-bound organelles or nucleus | Membrane-bound organelles and nucleus |
| **Metabolism** | Various, with methanogenesis unique to Archaea | Various, including photosynthesis, aerobic and anaerobic respiration, fermentation, and autotrophy | Photosynthesis and cellular respiration |

The classification of archaea is a rapidly moving and contentious field. Most of the culturable and well-investigated species of archaea are members of two main phyla, the Euryarchaeota and Crenarchaeota. The phylum Euryarchaeota encompasses the methanogens or methane producing Archaea and their phenotypically diverse relatives: the extreme halophiles, which are found in high salt concentration environments and some thermoacidophiles, which grow at a temperature exceeding 50°C and are isolated from volcanic terrestrial environments and deep-sea

hydrothermal vents. In contrast, the second phylum, Crenarchaeota contains only one taxonomic class composed exclusively of thermophiles or hyperthermophiles with an energetic metabolism mainly based on sulfur (Leigh et al., 2011).

Other groups of phyla have been tentatively created in the last few years. For example, the peculiar species Nanoarchaeum equitans, which was discovered in 2002, has been given its own phylum, the Nanoarchaeota (Huber *et al.,* 2002). A new phylum, the Korarchaeota, has also been proposed. It contains a small group of unusual thermophilic species that shares features of both the two main phyla, but it is more closely related to the Crenarchaeota (Barns *et al.,* 1996; Elkins *et al.,* 2008).

Based on the increasing wealth of whole genome data (mainly from the environmental isolates), the archaeal phylogeny has been revisited recently: the four groups Korarchaeota, Crenarchaeota, Thaumarchaeota (a widespread microbial group of mesophilic marine archaea) and the newly proposed Aigarchaeota have been comprised into one only superphylum (the so-called TACK-superphylum) to the exclusion of Euryarchaeota and Nanoarchaeota (Guy and Ettema, 2011). Whereas the carbohydrate metabolism of members of the Crenarchaeota and Euryarchaeota has been studied in considerable detail, only little information is available for the remaining archaeal phyla (Bräsen et al., 2014).

Methanogens are the most common and widely dispersed members of Archaea, being found in many environments and they are the only known microorganisms, which share a complex biochemistry for methane synthesis as the major end product of their energy metabolism.

## 2.2 Methanogens environmental distribution

Although all methanogenes share the same energy metabolism, they are morphologically and physiologically different. Morphologically, methanogens exhibit a wide variety of shapes and sizes, including rods, regular and irregular cocci, spirilla, pseudosarcina and plates (Garcia *et al.*, 2000). Also their distribution in the environment is entirely dependent on physical-chemical factors such as temperature, pH, and salinity range. Methanogens are found in mesophilic as well as in extreme environments. For example, methanogens include psychrophilic species growing at 1.7°C and extremely thermophilic species from deep submarine vents which are able to grow at 110°C, acidophiles species from marine vents growing at pH 5.0 and alkaliphiles species from alkaline lake sediments which grow at pH 10.3, other species growing at saline concentration below 0.1M and extreme halophiles which grow at nearly saturated NaCl concentrations (Sowers, 2009).

There are three different methanogenic ecosystems operating in nature (Figure 2.2-1) which reflect the physiological diversity of methanogens (Garcia *et al.,* 2000).

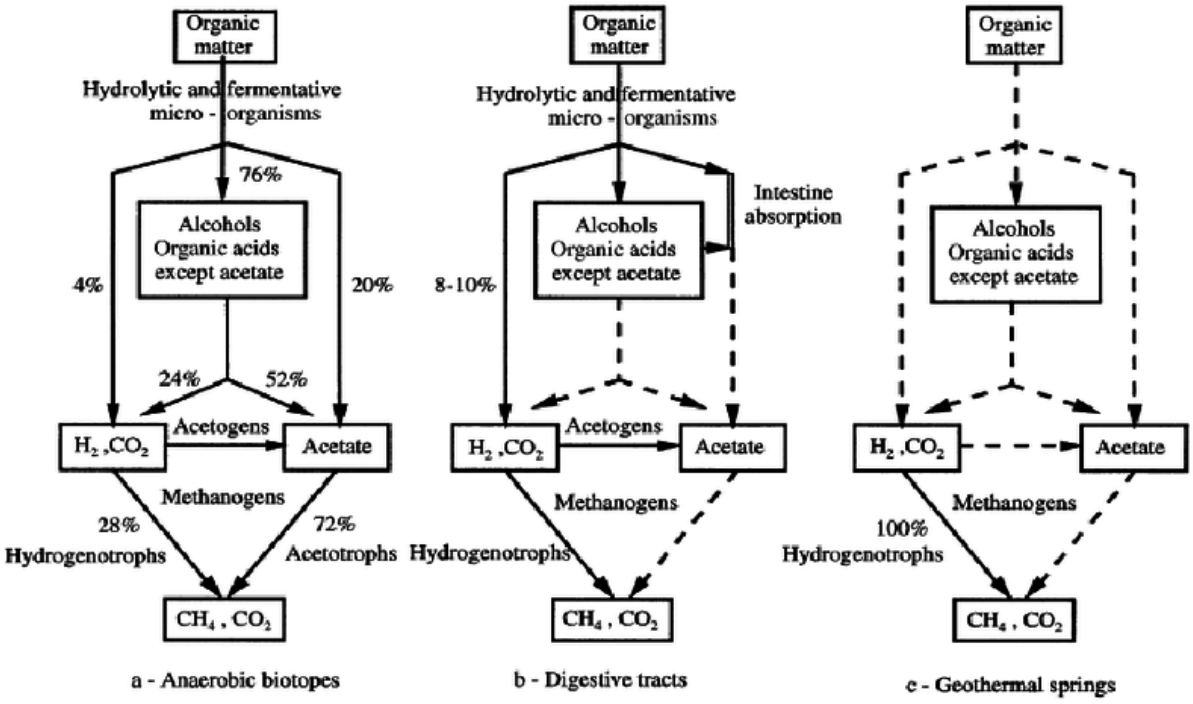


**Figure 2.2-1. Schematic diagram showing the three different methanogenic ecosystems operating in nature (Garcia *et al.,* 2000).**

a) Anaerobic environments, such as freshwater and marine sediments, marshes, rice soils, waste digesters, where the organic matter is completed degraded (Figure 2.2-1a). These enviromentes harbor a wide range of methanogens of the orders Methanosarcinales, Methanomicrobiales and Methanococcales (Chaban *et al.,* 2006).

b) Digestive tracts of different organisms, such as humans, ruminants, insects and termites, where the process of mineralization is incomplete and most of the intermediate products formed (e.g. volatile fatty acids) is resorbed into the bloodstream and serves as nutrition (Figure 2.2-1b). The Methanogenesis in the digestive tracts is mostly hydrogenotrophic and frequently includes methanogens of the order Methanobacteriales (Lange *et al.,* 2005).

c) Geothermal environments (e.g. hot springs), where the methanogenesis, in the absence of organic matter, occurs only from geochemical hydrogen formed as part of the geological process (Figure 2.2-1c). The Methanogens belonging to the orders Methanobacteriales, Methanococcales and Methanopyrales have been isolated from these environments (Jones *et al*.,1983; Lauerer *et al*., 1986; Kurr *et al*., 1991).

## 2.3 Methanogenesis

Methanogens are a group of microorganisms, which produce methane as a metabolic product under anaerobic conditions, and the methanogenesis is the only active metabolism by which methanogens can obtain the energy for growth. This energy derives from the conversion of a restricted number of substrates to $CH_4$. In the anaerobic breakdown of organic matter, methanogens occupy the terminal position and the major substrates which they can use are $H_2$, acetate and methylated compounds (such as methanol, methylated amines and methylated sulfides) which are directly produced by hydrolytic and fermentative bacteria (Garcia *et al*., 2000).

Therefore, there are three different methanogenic pathways that differ in their substrates: 1) the hydrogenotrophic methanogenesis, that use $H_2$ and $CO_2$ for methane synthesis; 2) the acetoclastic methanogenesis, in which the methyl group from acetate is transferred to tetrahydrosarcinapterin and then to coenzyme M (CoM); 3) the methylotrophic methanogenesis, using methyl groups from methanol and methylamines (mono-, di-, and trimethylamine) for the production of methyl-coenzyme M (Garcia *et al*., 2000).

Although the intermediates and the enzymatic reactions of the three pathways are different, they share common features in the final step of the $CH_4$ production. The final enzymatic step is catalyzed by the methyl-coenzyme M reductase (MCR) which is unique to methanogens. Hence, MCR constitutes a functional marker of microorganism involved in this metabolism and is used to investigate the diversity of the methanogenic communities.

### 2.3.1 The different pathways for $CH_4$ production

**Hydrogenotrophic methanogenesis** or **$CO_2$ reducing pathway** (Figure 2.3- 1) where the energy substrate is $H_2$, formate or certain alcohols and the electron acceptor is $CO_2$, which is reduced to methane (Rouvière and Wolfe,1988).

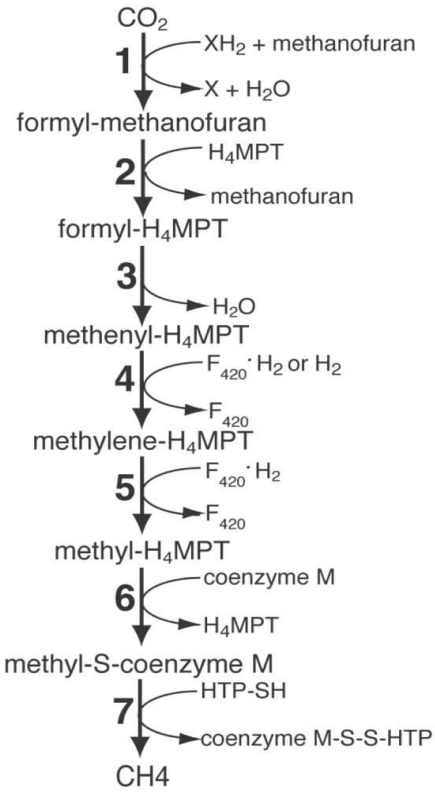


**Figure 2.3-1. Pathway of methanogenesis from H2 + CO2 which convert CO2 into methane** (Goldman *et al.,* 2009). In the initial step of methanogenesis from $H_2$ + $CO_2$, a two-electron reduction of $CO_2$ and methanofuran are catalyzed by formyl-methanofuran dehydrogenase and $CO_2$ is reduced to the formyl level (1); in the second step the formyl group is converted to formyl-tetrahydromethanopterin ($H_4$MPT) (2) and then (3) its cyclization is catalyzed to methenyl-$H_4$MPT. The methenyl-$H_4$MPT is reduced in two steps (4-5 and two electrons each) by the electron carrier coenzyme $F_{420}$ to methylene-$H_4$MPT and then methyl-$H_4$MPT by dehydrogenase and reductase enzymes. The methyl group is subsequently transferred to coenzyme M to form methyl-S-coenzyme M by methyltransferase (6). The last step (7) is the reduction of methyl-S-coenzyme M to $CH_4$by reductase enzyme which uses coenzyme M as an electron donor.

Most of methanogens can grow using $H_2$ as a source of electrons via hydrogenase and for some of them this is the only catabolic pathway, whereas some hydrogenotrophs use formate, which is formed from $CO_2$ and $H_2$ (Bleicher *et al*., 1989). $CO_2$ reduction is a very important reaction for maintaining a very low concentration of $H_2$ and formate is typical for the anaerobic habitats faciliting the process of interspecies electron transfer. The hydrogenotrophic methanogens belong to the orders Methanobacteriales, Methanococcales and Methanopyrales and to the orders Methanomicrobiales and Methanocellales (Bapteste *et al.,* 2005).

**Methylotrophic patways** catabolize compounds which contain methyl groups, such as methanol, methylamine, dimethylamine, dimethylsulfide, etc. which are used as electron acceptors and are reduced directly to $CH_4$. Typically the methyl group is transferred to a methyl carrier (ultimately to coenzyme M) and reduced to methane (Ferry, 2010). The methylotrophic methanogens are a phylogenetically and biochemically heterogeneous group comprising members of the order Methanosarcinales (Sprenger *et al*., 2000), the genus Methanosphaera and some species of the genus Methanobacterium (Fricke *et al.,* 2006), and members of the recently discovered seventh order of methanogens, the Methanomassiliicoccales (Paul *et al*., 2012; Borrel *et al*., 2014).

**Acetoclastic pathways** catabolize acetate molecules by reducing their methyl carbon to $CH_4$ and by oxidizing their carboxyl group to $CO_2$. This pathway is only performed by the members of two genera, Methanosarcina and Methanosaeta (both belonging to the order Methanosarcinales, Thauer *et al.,* 2008). Acetate is present in many environments and it is common in anoxic freshwater sediments in which the methyl carbon of acetate is reduced to methane and the carboxyl carbon is oxidized to $CO_2$ (Conrad, 1999).

The placement of most methanogens into three groups can be explained by the standard changes in free energies for methanogenesis (Table 2.3-1). The most favourable reaction is the reduction of $CO_2$ by $H_2$ and the least favourable is the acetoclastic reaction.

**Table 2.3-1. Reaction and standard changes in free energies for methanogenesis** (Whitman et al., 2006)

| Reaction | $\Delta G^{0,}$ (KJ/ml $CH_4$) |
|---|---|
| $CO_2$-type substrates | |
| $4\ H_2 + CO_2 \rightarrow CH_4 + 2H_2O$ | -135.6 |
| $4$ Formate $\rightarrow CH_4 + 3CO_2 + 2H_2O$ | -130.1 |
| $2$ Ethanol $+ CO_2 \rightarrow CH_4 + 2$ Acetate | -116.3 |
| Methyl substrates | |
| Methanol $+ H_2 \rightarrow CH_4 + H_2O$ | -112.5 |
| $4$ Methanol $\rightarrow 3CH_4 + CO_2 + 2H_2O$ | -104.9 |
| $4$ Methylamine $+ 2H_2O \rightarrow 3CH_4 + CO_2 + 4NH_4^+$ | -75.0 |
| $2$ Dimethylamine $+ 2H_2O \rightarrow 3CH_4 + CO_2 + 2NH_4^+$ | -73.2 |
| Acetotrophix substrate | |
| Acetate $\rightarrow CH_4 + CO_2$ | -31.0 |

## 2.4 Taxonomic diversity of the Methanogens

The current taxonomy of methanogens is based on the comparison of 16S rRNA gene sequences and a group of other minimal standards including morphology, Gram staining, electron microscopy, susceptibility to lysis, motility, colony morphology, grow conditions (temperature, pH, NaCl and culture medium), end-products, lipid analysis, G+C content of the DNA. Based on these criteria proposed by Boone and Whitman (1988), so far all known methanogens belonging to the phylum Euryarchaeota are classified into seven orders: Methanobacteriales, Methanococcales, Methanomicrobiales, Methanosarcinales, Methanopyrales, Methanocellales and Methanomasiliicoccales, which include 10 families and 31 genera (Liu and Whitman, 2008; Sakai *et al*., 2008; Paul *et al*., 2012; Lino *et al*., 2013). The seventh order of methanogens, Methanomassiliicoccales, was recently proposed on the basis of sequences retrieved from human gut (Mihajlovski *et al*., 2010). Two names were subsequently proposed, Methanoplasmatales (Paul *et al*., 2012) and later Methanomassiliicoccales (Lino *et al.,* 2013).

### 2.4.1 Order Methanobacteriales

The order Methanobacteriales is divided into two families, the Methanobacteriaceae and the Methanothermaceae, on the basis of the phylogenetic analysis of the 16S rRNA gene sequences as well as phenotypic characteristics (Boone and Castenholz, 2001).

The order Methanobacteriales is composed of rod-shaped and non-motile methanogens and the predominant cell wall polymer is pseudomurein. The Methanobacteriales are generally hydrogenotrophic, using $H_2$ to reduce $CO_2$ to $CH_4$. The exception is the genus Methanosphaera, which grows as cocci and uses $H_2$ to reduce methanol instead of $CO_2$.

#### *2.4.1.1 Family Methanobacteriaceae*

The family Methanobacteriaceae contains four morphologically distinct genera: Methanobacterium, Methanobrevibacter, Methanosphaera and Methanothermobacter.

The genus Methanobacterium includes 13 species. The type species is *Methanobacterium formicicum*. All species of this genus are rods, some are thermophilic with optimal growth temperature as high as 65°C and few are alcaliphilic. Only six species can use formate and three species can grow on 2-propanol/$CO_2$., while all species are able to grow on $H_2$ + $CO_2$ (Garcia *et al.,* 2000).

The genus Methanobrevibacter includes 14 species with different specialized habitats. *Methanobrevibacter ruminantium*, the type species, is the predominant methanogen of the bovine rumen, while *Methanobrevibacter smithii* is abundant in sewage sludge and digestive tracts of animals (Miller and Wolin, 1986). The Methanobrevibacter species are mesophilic.

The genus Methanosphaera comprises two species, *Methanosphaera stadtmane* and *Methanosphaera cuniculi*, which have been isolated from faeces of man and rabbit (Ferry, 2010). The members of this genus are spherical and mesophile methanogens requiring both methanol and $H_2$ as substrate for methanogenesis.

The genus Methanothermobacter is represented by the six species described like thermophilic and they generally grow optimally between 55°C and 65°C. All these species use $H_2$+$CO_2$ as substrate for methanogenesis and a few of them can use formate (Garcia *et al.,* 2000).

#### *2.4.1.2 Family Methanothermaceae*

The family Methanothermaceae consists of the single genus Methanothermus and two species, *Methanothermus fervidis* and *Methanothermus sociabilis* (Lauerer *et al.,* 1986). The members of this family are extreme thermofiles and have been isolated from thermal springs with temperatures of 85°C and pH 6.5. This species use only hydrogen and carbon dioxide as substrate for methanogenesis (Garcia *et al.,* 2000).

### 2.4.2 Order Methanococcales

The order Methanococcales is composed of irregular cocci and motile species which have been isolated from marine and coastal environments. They exhibit diversity in the temperature range for growth, which varies from mesophilic to hyperthermophilic. All the species use both $H_2$ and formate as electron donors, except the species of Methanocaldococcus, which are unable to utilize formate. Currently, the Methanococcales have been divided into two families and four genera (Garcia *et al.,* 2000).

#### *2.4.2.1 Family Methanococcaceaea*

The family Methanococcaceae contains two genera: Methanococcus and Methanothermococcus. The genus Methanococcus includes mesophilic species, with optimum temperature for growth between 35-46°C and pH between 6 and 8. The cells are slightly irregular cocci and the type species is *Methanococcus vannielii*.

The genus Methanothermococcus includes two moderately thermophilic species *M. okinawensis* and *M. thermolithotrophicus.* Their optimum temperature for growth is between 60-65°C (Sowers, 2009).

#### *2.4.2.2 Family Methanocaldococcaceae*

The two hyperthermophilic genera Methanocaldococcus, covering six species, and Methanotorris, covering two species, are placed in the family Methanocaldococcaceae. The cells are irregular cocci, motile by means of polar tufts of flagella. The optimum conditions for growth are: 85°C, pH between 5.7 and 6.5 and NaCl between 1.8 and 3.0 (Sowers, 2009).

### 2.4.3 Order Methanomicrobiales

The order Methanomicrobiales contains genera which are diverse in morphology and physiology. Most species grow as cocci, rods, and plane-shaped cells and all Methanomicrobiales can use $H_2 + CO_2$ as substrate for methanogenesis, most species can also utilize formate, and many species utilize alcohols. Like the methanogens belonging to other orders, Methanomicrobiales inhabit diverse anaerobic habitats comprising marine and fresh water sediments, anaerobic digesters and the rumen. Based upon the phylogeny of the 16S rRNA genes as well as phenotypic and genotypic characteristics, the order Methanobacteriales has been divided into three families and nine genera (Boone *et al.,* 1993; Garcia *et al.,* 2006) as described below.

#### *2.4.3.1 Family Methanomicrobiaceae*

The family Methanomicrobiaceae contains seven distinct genera with morphologies ranging from rods to irregular cocci or plane-shaped cells. The coccoid Methanomicrobiaceae have protein S-layers, which cause them to stain Gram-negative and to be osmotically fragile.

The most common genera are described below.

The genus Methanomicrobium is represented by a single mesophilic species, *Methanomicrobium mobile*. These Gram-negative and curved rods are motile and subject to frequent lysis. It was isolated from the bovine rumen and it has a complex nutritional requirement, which includes rumen fluid or a mixture of volatile fatty acids (Garcia *et al*., 2000). It produces methane from $H_2 + CO_2$ or from formate.

The genus Methanogenium contains five species isolated from various environments and ranging from psychrophile to thermophilic. Morphologically they are irregular cocci, stain Gram-negative and are non-motile (Garcia *et al*., 2000).

The genus Methanoculleus consists of five mesophilic species of irregular non-motile cocci and one thermophilic species. The formate is used by five species (Garcia *et al*., 2000).
The genus Methanoplanus comprises three species of plane-shaped organisms. The type species is *Methanoplanus limicola*, which uses formate for the methanogenesis.

#### *2.4.3.2 Family Methanocorpusculaceae*

The family Methanocorpusculaceae contains one genus, Methanocorpusculum, and five mesophilic species of irregular cocci. The optimum conditions for growth are 37°C and pH 7. They use $H_2$ + $CO_2$ and formate, and some species can use 2-propanol + $CO_2$ for the methane production. The type species is *Methanocorpusculum parvum*, which is of special interest because it requires high levels of tungstate for growth (Zellner *et al.,* 1987).

#### *2.4.3.3 Family Methanospirillaceae*

The family Methanospirillaceae includes the single genus Methanospirillum (Boone *et al.,* 1993). The members of the genus are mesophilic and have been reported from a wide range of habitats. However, only one species, *Methanospirillum hungatei*, has been described so far (Ferry *et al.,* 1974). The cells are curved rods and often form filaments several hundred µm in length (Garcia *et al*., 2000). The type species forms methane from $H_2$ + $CO_2$ or from formate. No growth or methane production is detected on acetate, methanol or ethanol.

### 2.4.4 Order Methanosarcinales

The order Methanosarcinales includes the most catabolically diverse species of methanogens. Many of them can grow by reducing $CO_2$ with $H_2$, some species can also grow by methyl reduction with $H_2$, or by the splittng of acetate. Some species can use only one of those catabolic pathways, but the others can use all the three. The members of the order Methanosarcinales are coccoids, pseudosarcinaea, or sheathed rods and all species have a protein S-layer cell wall. Similarly to the other methanogens, Methanosarcinales are widespread in diverse anaerobic habitats, including freshwater and marine sediments, anaerobic sludge digestors and the gastrointestinal tracts of animals. The order Methanosarcinales comprises two families, Methanosarcinaceae and Methanosaetaceae (Garcia *et al.,* 2000).

### *2.4.4.1 Family Methanosarcinaceae*

The family Methanosarcinaceae includes six genera of coccoidal or pseudosarcinal bacteria. The following genera, except the genus Methanosarcina, are obligatory methylotrophic methanogens which have been isolated from environments with high salt concentration.

The genus Methanosarcina comprises nine species. *Methanosarcina barkeri* is the type species and the most studied acetoclastic methanogen. The genus Methanosarcina represents the acetotrophic methanogens, which predominate in many anaerobic ecosystem and are found in freshwater and marine mud, anoxic soils, animal-waste lagoons and anaerobic digestors, where organic matter is completely degraded to $CH_4$ and $CO_2$. Some of these are the most versatile methanogens (Garcia *et al.,* 2000), they are able to use $H_2 + CO_2$, actate and methyl compounds (methanol, methylamines).

The genus Methanolobus is represented by five species with coccoidal morphology. The type species, *Methanolobus tindarius*, is an irregular mesophilic coccus isolated from coastal sediments and the optimal concentration of NaCl is about 0.5 M.

The genus Methanococcoides includes two species with *Methanococcoides methylutens* as the type species. The cells are irregular cocci, and can dismutate methylamines and methanol for growth, but they are not able to catabolize acetate, dimethylsulfide, $H_2/CO_2$ or formate. The optimal concentration of NaCl is 0.2-0.6 M and high concentration of magnesium is also required.

The genus Methanohalobium contains a single species, *Methanohalobium evestigatum*, which is extremely halophilic and moderately thermophilic, and grow at 25% NaCl at 50°C. Methanolobium are generally found in extremely hypersaline environments.

The genus Methanohalophilus comprise four species, which are mesophilic and hyperhalophilic species. *Methanohalophilus mahii* is the type species. The cells are non-motile and can grow only on methylamines and methanol. The optimum salinity for growth is 1-2.5 M NaCl.

The genus Methanosalsum is represented by only one alkaliphilic and halophilic species, *Methanosalsum zhilinae*. Energy and methane production are possible when this strain is grown on methylamines, methanol or dimethylsulfides, but not on acetate, formate or $H_2/CO_2$ (Boone *et al.,* 1986; Kadam and Boone, 1996).

### *2.4.4.2 Family Methanosaetaceae*

The family Methanosaetaceae includes one genus, Methanosaeta, that is represented by two species. *Methanosaeta concilii* is the type species, with rod-shaped cells which form an immunologically cohesive group. All the cells utilize acetate as the sole energy substrate for

methanogenesis. The members of the genus Methanosaeta are found in anaerobic digestors and sediments.

### 2.4.5 Order Methanopyrales

The order of Methanopyrales is represented by only one family Methanopyraceae, one genus Methanopyrus and one species *Methanopyrus kandleri*, which is hyperthermophilic (grow at 110°C) and produces methane by $CO_2$ reduction with $H_2$. The cells are rod-shaped and stain Gram-positive (Garcia *et al.,* 2000). The cell wall is double layered and the inner layer is composed of a new type of pseudomurein, containing ornithine and lysine. It has been isolated from hydrothermally heated deep sea sediment and from shallow marine hydrothermal system (Kurr *et al.,* 1991).

### 2.4.6 Order Methanocellales

The Methanocellales represents a new order of methanogens (Sakai *et al*., 2008), which is widespread in the environments. Methanocellaceae is the only family within the Methanocellales order, which includes only one genus Methanocella with species arvoryzae, conradii and paludicola. The last one is type species. The cells are stationary, irregular and anaerobic rods. As substrate for methanogenesis this methanogen uses $H_2$ and $CO_2$.

### 2.4.7 Order Methanomassiliicoccales

The order Methanomassiliicoccales contains only one family Methanomassiliicoccacee and one genus Methanomassiliicoccus, which comprises only the type species *Methanomassiliicoccales luminyensis* (Borrel *et al.,* 2014). The cells are irregular cocci and members of this order have been found in marine and soil environments, as well as from subgingival pockets and the intestine of termites and mammals, including the human gut (Dridi *et al.,* 2012; Borrel *et al.,* 2013; Söllinger *et al.,* 2016). The members of this order are strictly anaerobic, obligate $H_2$-dependent methanogens. One of the few general characteristics of the order is that the production of methane is dependent on hydrogen and methanol and methylamines. All the strains lack the genes encoding the entire $C_1$ pathway for the reduction of $CO_2$ to methyl-CoM, but possess the complete gene sets for the utilization of methanol and methylamines (Borrel *et al.,* 2013). This explains the strict dependence of methanogenesis on the simultaneous presence of hydrogen and methanol or trimethylamine documented for *Methanomassiliicoccales luminyensis* (Lang *et al.*, 2015).

## 2.5 Detection of the methanogen diversity

The DNA sequence analyses of marker genes are considered as a potential strategy to study bacterial phylogeny and diversity (Tringe and Hugenholtz, 2008). Before the introduction of molecular methods, the studies on methanogenic archaea were based on their bacteriological isolation and quantification. These studies were mainly focused on physiology, morphological features (such as capsules, flagella, cell size and shape), metabolic characterization (e.g. lipid composition, substrates required for growth) and methanogenesis pathways (Saengkerdsub and Ricke, 2013) and these properties have been used for the identification and classification of the archaeal species. In the past twenty years, the molecular approaches have provided an alternative means for describing microbial diversity, increasing the number of recognized prokaryotic phyla (Hugenholtz *et al*., 1998, Rappe and Giovannoni, 2003), and revealing novel methanogenic archaea, such as a wide diversity of mesophilic Archaea with unknown function (Schleper *et al*., 2005). The methanogen communities have been characterized by employing the 16S ribosomal RNA (rRNA) gene or the methyl-coenzyme M reductase gene (mcrA) as molecular markers in a wide variety of environments and biodiversity studies have relied on the use of those two genes as molecular markers for the detection and phylogenetic analysis of methanogens.

### 2.5.1 Ribosomal 16S rRNA gene as molecular marker

The small subunit ribosomal RNA (16S rRNA) gene sequence analysis is used for the identification and classification of prokaryotes. Currently, the 16S rRNA gene is the best target for phylogenetic studies for two reasons: 1) it is universally present in bacteria and archaea; 2) the function of the 16S rRNA gene over time has not changed, containing a mosaic structure of highly conserved and more variable domains, which are species-specific (Patel, 2001). The conserved sequence regions allow design of primers for different taxonomic levels, and interspersed variable regions and the length of the gene (1500 bp) provide phylogenetic resolution for distinguishing taxa (Figure 2.5-1). Numerous methods based on the 16S rRNA genes have become standard for conducting microbial studies. A standard approach in molecular analysis of microbial communities starts with the extraction of DNA from the environmental samples, followed by PCR amplification of marker genes, using specific primers, differentiation of amplicons by cloning and identification of the populations by sequencing and phylogenetic analysis (Head *et al*., 1998).

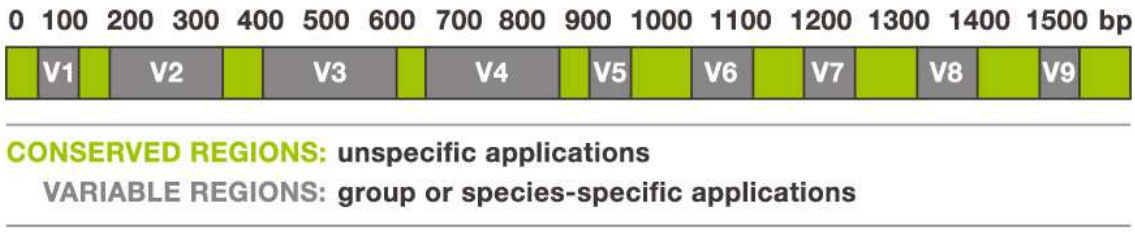


**Figure 2.5- 1: 16S rRNA gene illustrating the conserved (green) and variable (grey) regions (Lu Cheng, 2013).**

The 16S rRNA clone libraries have been applied to the GI tract studies due to commercially available cloning vectors and numerous of the 16S rRNA sequences are available in GenBank. The widespread application of the 16S rDNA as a molecular marker in microbial ecology has been central to discovery of numerous novel prokaryotic lineages (Hugenholtz *et al.,* 1998; Rappe and Giovannoni, 2003). A set of universal primers binding to the conserved regions can be used for the identification and this approach has become a useful phylogenetic tool (Woese *et al*., 1990).

In addition to the sequence analysis, DNA fingerprinting analysis based on 16S rRNA genes such as denaturing gradient gel electrophoresis (DGGE), amplified ribosomal DNA restriction analysis (ARDRA) and terminal restriction fragment polymorphism analysis (T-RFLP) are widely used to differentiate microorganisms (Bouchet *et al.,* 2008). All these methods are based on the sequence variations in the 16S rRNA genes. In the DGGE method, DNA targets are amplified by PCR and the PCR product up to 500 bp in length are separated in a polyacrylamide gel containing a linear gradient mixture of urea and formamide (Hanning and Ricke, 2011).

### 2.5.2 The gene *mcrA* as specific marker of Methanogens

The Methyl-coenzyme M reductase (MCR) is an essential enzyme in $CH_4$ production. It catalyzes the final step of methanogenesis, in which the methyl group linked to coenzyme M is reduced with the formation of $CH_4$ (Ellermann *et al.,* 1988, Deppenmeier, 2002). This enzyme is present in all known methanogens and, unlike many other enzymes in the methanogenic pathway, it is absent in non-methanogenic Archaea and Bacteria (Bapteste *et al*., 2005). This

enzyme complex is thought to be unique and ubiquitous in methanogens, making it a suitable tool for their specific detection.

MCR is composed of three subunits, α, β, and γ, encoded by the operon mcrBDCGA (Reeve *et al*., 1997). The gene encoding the α-subunit, *mcrA*, contains conserved sequence regions, which have been related to catalytic sites of MCR (Weil *et al.,* 1988, Hallam *et al.,* 2003). The phylogeny of the *mcrA* follows the 16S rRNA phylogeny (Springer *et al*., 1995, Luton *et al.,* 2002), allowing the identification of methanogens based on mcrA sequences. Several primer pairs have been designed for the detection of the *mcrA* gene (Ohkuma *et al*., 1995; Springer *et al*., 1995; Hales *et al*., 1996; Luton *et al*., 2002). The primers differ in amplicon length, target site, and the level of degeneracy.

## 2.6 Methanogenic Archaea in the gastrointestinal tracts of different animals

Methanogens inhabit a large variety of environments, such as wetlands, sediments, digesters, geothermal springs and hydrothermal vent sites, as well as the digestive tract of animals and humans (Garcia *et al*., 2000).

In the gastrointestinal tract (GIT) of animals, fermentation of feed is implemented by a combined activity of an anaerobic complex of microorganisms, consisting of bacteria, protozoa, fungi and archaea, which are mutually dependent through complex trophic relationships (Castillo-González *et al.*, 2014). As a result of these activities, macromolecules such as polysaccharides, proteins and lipids are metabolized into smaller organic compounds, such as volatile fatty acids (VFAs), which are absorbed by the host to provide energy. Other products of microbial anaerobic respiration, including carbon dioxide and methane, are not assimilated by the host and are released into the environment.

The methane emissions from livestock are problematic for two reasons. First, the enteric production of methane has a negative impact on animal feed efficiency, resulting in lost energy ranging from 2% to 12% of the animal's gross energy intake (Pierre and Wright, 2012). Second, once released into the environment, the methane acts as a potent greenhouse gas with 25 times the global warming potential of carbon dioxide (Pierre and Wright, 2012).

Although they do not contribute to fulfilling their host's energy requirements, methanogens play an important role in the GIT of herbivores by maintaining the fermentative performance of the

microbial community. By metabolizing the $H_2$ generated from the fermentation of the plant polysaccharides, methanogens function as a sink to maintain a low $H_2$ pressure, which promotes plant fiber digestion by protozoa and bacteria (Wolin *et al.*, 1997). In ruminants, the fermentation products provide the animals with 70% of their energy whereas bacteria in hindgut generate 10-20% energy to the host human and swine (Jòzefiak *et al.,* 2004). Also the equine large intestine is a fermentative chamber, which contains an extremely abundant and diverse community of anaerobic microorganisms. The equine hindgut intestinal microbial community of similar complexity as ruminants is assumed to contribute as much as 63% to 68% to total energy, by producing short chain fatty acids (Vermorel *et al.,* 1997). This high amount of energy retreaved by the equidae from the end microbial fermentation products, points out the significance of equine caeco-colic microbiota.

Methanogens have been isolated from various animals and several studies using culture-independent methods, including 16S rRNA gene clone library analysis, have provided some useful data on the diversity and abundance of methanogens in rumen (Wright *et al*., 2004; Wright *et al*., 2007; Pei *et al*., 2010) and recently also in equine hindgut (Lwin and Matsui, 2014; Fernandes *et al*., 2014).

The 16S rRNA gene is the most commonly used phylogenetic marker for the characterization of bacterial and methanogen communities in the GIT of animals (Skillman *et al.*, 2006; Rajendhran and Gunasekaran, 2011). These studies indicated that methanogen diversity in the GIT may be host species-specific and/or function-dependent.

The physiological characteristics of methanogenic archaea residing in the animal GI tracts are similar. The optimal growth temperature range of the majority of methanogens is between 35 and 42°C and the optimal pH is approximately 7.

Based on the methanogenic strains identified by sequencing 16S rRNA genes, the major groups of methanogens which have been identified in the GIT of herbivores belong to the genus Methanobrevibacter (order Methanobacteriales, Garcia *et al*., 2000) and the dominance of Methanobrevibacter-related archaea is reported by a number of different studies. However, GIT Methanobrevibacter-related methanogens from livestock animals tend to be more closely related to either *Methanobrevibacter ruminantium, Methanobrevibacter millerae, Methanobrevibacter gottschalkii*, or *Methanobrevibacter smithii*, whereas *Methanobrevibacter woesei* related methanogens have only been reported in chickens. All *Methanobrevibacter spp.*, except the type strain *Methanobrevibacter arboriphilus* originating from decaying cottonwood tissue, were isolated from GI tracts (Miller and Lin, 2002; Rea *et al.,* 2007).

Although the genus Methanobrevibacter is also the primary methanogenic group reported in the herbivore GIT samples, other genera have been isolated or found. Indeed, the members of the order Methanomassiliicoccales (Lino *et al*., 2013) are also a prominent group of GIT methanogens. The rumen methanogens from this taxonomic group have been reported to use methylamines as substrates for methanogenesis (Poulsen *et al.,* 2013). While they are, in general, less abundant than Methanobrevibacter-related or Methanomassiliicoccales sequences, 16S rRNA gene sequences which are more closely related to other methanogen species, such as *Methanosphaera stadtmanae* and *Methanomicrobium mobile*, or genera, such as Methanoculleus and Methanosarcina, have also been identified in the GIT of herbivores. While they are usually detected at a low frequency, in some studies they have been shown to be the most prevalent methanogens under certain conditions. Previous studies have shown that *Methanomicrobium mobile* is the predominant methanogen in the ovine rumen (Yanagita *et al.,* 2000) and in the rumen of Murrah buffaloes (Chaudhary and Sirohi, 2009). *Methanosphaera stadtmanae* was found to be the most prevalent methanogen in the hindgut of captive orangutans (Facey *et al.,* 2012). This methanogen species has a limited substrate range for methane synthesis, and is notably unable to use $H_2$ and $CO_2$ for this purpose. Furthermore, archaea belonging to the order Methanomicrobiales were predominant in the GIT of Japanese local ponies and thoroughbred horses (Lwin and Matsui, 2014). Finally, *Methanocorpusculum labreanum* was found to be the most abundant (59.9%) in the hindgut of captive white rhinoceroses (Luo *et al*., 2013). The identification and predominance of this type of methanogen in a GIT environment is unusual compared to most other reported studies.

The molecular approaches have revealed that the composition of methanogens in animal intestinal tracts can vary with different diets. It was reported by Wright et al. (2004) that sheep fed with pasture-grazed exhibited greater methanogen diversity than sheep fed either oat hay or lucerne hay. An increase in the fiber content of diets has shown an increasing availability of methanol and methylamine in the GI tract. Diets with a higher fiber level usually trigger more $CH_4$ emission per unit food than diets with lower fiber levels (Beauchemin *et al.,* 2008; Hristov *et al.,* 2013). Pol and Demeyer (1988) demonstrated that sheep fed a hay-concentrate diet increased the rate of methanogenesis from methanol, which is a substrate for *Methanosarcina spp.*

# OBJECTIVE

The objective of the present thesis included the description and comparison of the diversity of the archaeal community in the six different parts of the large intestine of the horse and of the donkey by the methods of molecular biology with the aim to elucidate the physiological and symbiotic role of these microorganisms in the digestion processes. The general target of this work is to contribute improving the limited knowledge about the microbiology of the equine hindgut system and to better understand the role of the different gastrointestinal segments in the feed fermentation. The gut microbiota is complex ecosystem in many mammals and detailed knowledge of the gut bacteria communities are essential for maintaining gut homeostasis as well as for the prevention and treatment of intestinal diseases.

# 3. MATERIALS AND METHODS

## 3.1 Animals and diets

The samples for this study were taken from one adult (24 years old) Anglo-Arabian castrated male horse and from one female donkey (8 years old) reared in two different farms in Sardinia (Italy). The horse (410 kg live-weight) housed in stall bedded with wood shavings, regularly vaccinated and dewarmed, was offered by meadow hay (5.4 kg of DM/day) and concentrate (1.6 kg of DM/day) divided into two meals. The concentrate represented complementary feed and its composition is reported in the table 3.1-1; it was composed of: corn, dry pellet of alfalfa, peas, corn ground, wheat bran, cocoa peel, sunflower seed, corn flakes, barley flakes, pea flakes, soya bean, molasses of sugar cane, calcium carbonate, sodium chloride, sodium bicarbonate as described in Mura (2013). The donkey (210 kg live-weight), in the last 4 months before sampling, was on continuous grazing, feeding (estimated feed intake 3.4 kg DM/day) only on pasture of natural permanent meadow, with not any supplementation necessity. The animals were allowed ad libitum access to water. Both the animals were clinically healthy and did not show any clinical sign of disease or intestinal disturbances within the previous 6 months.

**Table 3.1-1: Composition complementary feed (Mura, 2013)**

| **Analytic Components%** | | **Additives per Kg:** | | **Oligo-elements:** | |
|---|---|---|---|---|---|
| Moisture | 12,50% | Oxide of Magnesium | 60 mg | Iron | 15,2 mg |
| Crude proteins | 14,50% | Vitamin A E 672 | 4800 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-Pantoteniv | 0,48mg | | |

## 3.2 Collection of gut samples

The samples of the luminal gut contents were collected from three different regions of the horse and donkey large intestine: caecum (Ce), colon (C1 – C4) and rectum (Fe). In particular, from the colon the samples of right ventral colon (RVC, C1), left ventral colon (LVC, C2), left dorsal colon (LDC, C3) and right dorsal colon (RDC, C4) have been taken. The samples were collected immediately after the euthanasia (within 20-30 minutes), the content of each region of the gastrointestinal tract was placed into a clean bag under vacuum seal and transported on ice in the laboratory, where all the samples were freeze-dried and stored at -20$^{o}$C until required for the analysis. The samples were weighed before and after the lyophylization, to determine the percentage of moisture and dry matter contents. The horse and donkey samples were distinguished by letters H and D as described in the table 3.2- 1.

**Table 3.2-1: Prefixes used to distinguish the different regions of the horse and donkey hindgut**

| Part of GIT | HORSE | DONKEY |
|---|---|---|
| Caecum | HCe | DCe |
| Right ventral colon (colon1) | HC1 | DC1 |
| Left ventral colon (colon2) | HC2 | DC2 |
| Left dorsal colon (colon3) | HC3 | DC3 |
| Right dorsal colon (colon4) | HC4 | DC4 |
| Rectum (faeces) | HFe | DFe |

## 3.3 DNA extraction

The DNA extraction was carried out from the lumen content of the two species using two different methods, in relation to the nature of samples. Those from the horse were represented by voluminous, long stems of rest of food and the DNA was isolated using the modified method of Gardes and Bruns (1993). While the samples of the donkey were represented by much more fine and fragmented material and the DNA was isolated directly using PowerPlant® Pro DNA Isolation kit, which is faster and more simple.

### 3.3.1 Horse: DNA extraction using CTAB method

The nucleic acids were extracted from each intestinal sample of the horse using the modified method of Gardes and Bruns (1993). Five g of freeze-dried gut content were disrupted by mortar and pestle after pouring of liquid nitrogen on the sample. The genomic DNA was isolated from 400 mg of the disintegrated powder, using the cetyltrimethylammonium bromide extraction protocol. The concentration and quality of the nucleic acids were checked by NanoDrop 2000c UV-Vis spectrophotometer (Thermo Scientific, U.S.A) and the DNAs were stored at -20°C until required. The detailed DNA isolation procedure is described below:

1. Transfer 400 mg of sample pulverised under liquid nitrogen in a chilled pestle and mortar into a 1.5 ml tube.
2. Add 3 ml of CTAB extraction buffer and incubate at 65°C for 60 minutes.
3. Gently mix by inverting the microtube from time to time.
4. Add an equal volume of chloroform to the DNA sample (1:1) and mix vigorously by vortex or inversion.
5. Centrifuge for 10 min at 12 000 rpm and 20-25°C to separate the aqueous and organic phases. Transfer the upper aqueous phase to a clean tube (then discard the rest).
6. Repeat this extraction step (steps 4-5) two or three times.
7. Transfer upper aqueous phase to a new tube and add 0.1 volume of 3 M sodium acetate and 2.5 volumes of 96% cold ethanol, mix gently and incubate the tube overnight at -20°C to precipitate the DNA .
8. To pellet the DNA, centrifuge the tube at 11000 rpm for 15 min at 4°C.
9. Rinse the DNA pellet twice with 70% ethanol and remove residual ethanol by vacuum.
10. Resuspend DNA in 1 ml TE.
11. Transfer 200µl of sample in new tubes with 1 ml of the Bind buffer and add 10µl of glass milk and mix well.
12. Centrifuge for 30 sec at 11 000 rpm and discard the supernatant solution.
13. Add 500µl of Neet wash and centrifuge for 30 sec at 11 000 rpm.
14. Repeat the Neet wash step two times.
15. Discard the supernatant solution.
16. Add 50 µl of TE buffer to release the DNA into solution.
17. Centrifuge at 11 000 rpm for 30 sec.
18. Transfer the supernatant solution containing the cleaned DNA in the new tube.
19. Store DNA at -20°C.

**Reagents and solutions**

*CTAB buffer 2%:*
2% CTAB (cetyltrimethylammonium bromide)
100 mM Tris-HCl (pH 8.0)
20 mM EDTA
1.4 M NaCl

*TE buffer:*
10 mM Tris-HCl pH 7.6-8.0 (25°C)
0.1 mM EDTA

*BIND buffer( pH 6.0):*
NaCl to saturate the buffer
10 mM Bis-Tris

*NEET wash( pH 7.5):*
100 mM NaCl
1 mM EDTA
10 mM Tris-HCl
50% EtOH

### 3.3.2 Donkey: DNA extraction using PowerPlant® Pro DNA Isolation kit

The DNA extraction from donkey samples, was carried out using the PowerPlant® Pro DNA Isolation kit (MO BIO Laboratories, Inc., USA). The detailed description of DNA isolation procedure is described below:

1. Add 50 mg of sample to the 2 ml provided Powerplant Bead Tubes and add 450 µl of Solution PD1.
2. Add 50 µl of Solution PD2 and 3µl of RNase A solution and vortex briefly to mix.
3. Homogenize sample using Powerlyzer™ 24 Homogenizer two times for 1 minutes.
4. Centrifuge Bead Tubes at 13 000 rpm for 2 minutes.
5. Transfer the supernatant to the clean 2 ml collection tube (provided).
6. Add 175 µl of Solution PD3. Vortex 5 seconds. Incubate at 4°C for 5 minutes.
7. Centrifuge the collection tube for 2 minutes at 13 000 rpm.
8. Avoiding the pellet, transfer up to 600 µl of supernatant to the clean 2 ml collection tube.
9. Add 600 µl of Solution PD4 and 600µl of Solution PD6. Vortex to mix for 5 seconds.
10. Load approximately 600 µl of lysate into the Spin Filter Tube and centrifuge at 10 000 rpm for 30 seconds. Discard the flow through, place the Spin Filter Tube back into the collection tube and add another 600 µl of lysate and centrifuge at 10 000 rpm for 30

seconds. Discard the flow-through and repeat for the third time until all of the lysate has been passed through the spin filter. Discard the flow-through and place the Spin Filter Tube back into the collection tube.

11. Add 500 µl of Solution PD5 to the Spin Filter Column. Centrifuge for 30 seconds at 10 000 rpm. Discard the flow-through. Place the Spin Filter Tube back into the same collection tube.
12. Add 500 µl of Solution PD6 to the Spin Filter Column. Centrifuge for 30 seconds at 10 000 rpm. Discard the flow-through. Place the Spin Filter Column back into the same collection tube.
13. Centrifuge again for 3 minutes at 13 000 rpm to remove residual Solution PD6.
14. Carefully place the Spin Filter Column into the new clean 2 ml collection tube. Avoid splashing any Solution PD6 into the Spin Filter.
15. Add 50 µl of Solution PD7 (10mM Tris, pH 8.0) to the center of the white filter membrane and incubate for 2 minutes at room temperature.
16. Centrifuge 1 min at 10 000 rpm.
17. For maximum elution efficiency re-load the flow through once again to the center of the white filter membrane. Centrifuge 1 min at 10 000 rpm.
18. Discard the Spin Filter Column.
19. Store DNA Store DNA at -20$^{o}$C.

## 3.4 DGGE analysis of Archaea

### 3.4.1 PCR Amplification of archaeal DNA fragment for DGGE analysis

The amplicons for DGGE study of archaeal profiles in different parts of horse and donkey digestive tract were prepared by nested PCR approach. First, the archaeal 16S rDNA was amplified from each sample using the specific primer pair Met86F (5´- GCTCAGTAACACGTGG- 3´) and Met1340R (5´- CGGTGTGTGCAAGGAG- 3´) according to Wright and Pimm (2003).The PCR reaction with primers Met86F and Met1340R was performed using PPP Master Mix kit (Top-Bio, Czech Republic). Each 30 µl PCR mixture contained 75 mMTris-HCl (pH 8.8), 20 mM $(NH4)_2SO_4$, 0.01% Tween 20, 2.5 mM $MgCl_2$, 200 µM dATP, 200 µM dCTP, 200 µM dGTP, 200 µM dTTP, 50 U/ml Taq DNA polymerase, 0.3 µM of each primer and 2µl of template DNA (10-fold diluted). The following PCR assay was

performed: 4 minutes of denaturation step at 94°C, 33 cycles consisting of 1 min at 94°C, 30 seconds at 58°C, 45 seconds at 72°C and final elongation step at 72°C for 2 min (Wright and Pimm, 2003).

These PCR products were purified by Qiagen PCR purification kit (Germany) as described below and used for the nested PCR amplification with the special DGGE primers PARCH340fGC (primer 5´-CCCTACGGGG(C/T)GCA(G/C)CAG-3´) with GC clump (5´-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG-3′) and PARCH519r (5′-TTACCGCGGC(G/T)GCTG-3´) (Øvreås et al.1997). The nested PCR reaction with these primers was performed using PPP Master Mix kit (Top-Bio, Czech Republic). Each 30 µl PCR mixture contained 75 mMTris-HCl (pH 8.8), 20 mM $(NH4)_2SO_4$, 0.01% Tween 20, 2.5 mM $MgCl_2$, 200 µM dATP, 200 µM dCTP, 200 µM dGTP, 200 µM dTTP, 50 U/ml Taq DNA polymerase, 0.3 µM of each primer and 1 µl of template DNA (10-fold diluted). The following PCR assay was performed: 2 minutes of denaturation step at 92°C, 33cycles consisting of 1 min at 92°C, 30 seconds at 55°C, 1 min at 72°C and final elongation step at 72°C for 6 min.

### 3.4.2 DGGE analysis

The DGGE analysis was performed according to the Muyzer *et al.* (1993) on DCode Mutation Detection System (BioRad Laboratories Ltd, Germany) on a 9% polyacrylamide gel with 35-60% denaturing chemical concentration (100% denaturant according to 7M urea and 40% formamide in 1X TAE-buffer). The electrophoresis was operated for 18 hours at 55 V and 60°C. The gel obtained from the electrophoresis was stained for 30 minutes in 1x TAE buffer with Gel Green Dye (0.001%) and visualized using a UV transilluminator (VilberLourmat, France). The following equipment and solutions were used:

#### 3.4.2.1 Basic equipment for DGGE

1. Two glass plates approximately 18x20 cm, one “eared” and the other plain
2. Spacers and combs 1mm thick
3. DGGE holder for stabilizing glass plates during gel making
4. Gradient maker, for small volumes (up to approximately 25 ml/chamber), and appropriate tubing
5. Magnetic stirrer and small magnets to fit diameter of gradient maker chambers

6. Tape
7. DGGE holder with electrodes (gel electrophoresis cassette)
8. Transparent aquarium with lid (to avoid excessive evaporation of buffer during the electrophoresis)
9. Heating element with thermostat and circulating ability
10. Thermometer to monitor temperature during the electrophoresis
11. Power supply
12. Fine-ended tips for sample loading
13. Staining tray
14. UV transilluminator, 256 nm wavelength
15. Camera

#### 3.4.2.2 Preparation of DGGE gel

1. Clean the glass plates well, using in succession strong detergent, water and finally acetone or alcohol. Dry well.
2. Place the spacers between the plates and tape the sides and bottom, sealing very well to prevent leakage.
3. Place the comb in between the glass plates and put the construction into a "holder" that stabilizes the plates in a vertical position. Suitable size of glass plates is approximately 18-20 cm, with spacers and combs 1 mm thick.
4. Connect the tubing to a clean and dry gradient maker, closing the communicating channel between the 2 chambers and also the tubing that leads from the gradient maker to the glass plates, with the use of stop-cock or clamps.
5. Position the magnetic stirrer about 25 cm above the glass plates and place the gradient maker on top, securing well.
6. In two separate test tubes prepare two denaturing solutions (representing the high and low of the range appropriate for the fragment).
7. Put the solution with the lowest denaturant concentration in the chamber of the gradient maker furthest from the glass plates and then place the solution with the highest denaturant concentration in the chamber nearest the glass plates.
8. Put the magnet in this chamber, turn stirrer on and open the connecting tube between chambers to begin mixing the two solutions. Immediately open the connection on the tubing leading to the glass plates and the acrylmide will begin to flow steadily under the force of gravity.

9. As soon as the solution has reached the comb, stop the flow and remove the tubing.
10. The gel will polymerise within 30-45 minutes, depending upon the ambient temperature.

### 3.4.2.3 Reagents and solutions for the preparation of one or two DGGE gels

| **Reagents** | **Amount (ml)** | | | |
|---|---|---|---|---|
| | 1 gel (25ml) | | 2 gels (50ml) | |
| | Denaturation rate | | | |
| | 35% | 60% | 35% | 60% |
| 40% acrylamide (ml) | 5.56 | 5.56 | 11.12 | 11.12 |
| 50x TAE buffer (ml) | 0.5 | 0.5 | 1 | 1 |
| Formamide (ml) | 3.5 | 6 | 7 | 12 |
| Urea (ml) | 3.68 | 6.1 | 7.35 | 12.6 |
| Deionized water (ml) | 12.25 | 9.5 | 25.5 | 19 |

1 x TAE buffer (pH 8):
40 mM Tris
20 mM acetic acid
1 mM EDTA

### 3.4.2.4 Electrophoresis of the DGGE gel

1. Fill the electrophoresis tank with running buffer (1x TAE). Heat buffer to 60 $^{o}$C.
2. Once the gel has polymerised, carefully remove the comb,remove the tape from bottom of plates.
3. Place the gel in the gel electrophoresis cassette and submerge in the buffer in the tank. Attach the electrophoresis cables.
4. Flush the wells with a fine syringe and load about 30 µl of each sample, containing loading dye, using thin-ended tip.
5. Run the gel about 18 hours (overnight) at 55 volts. The gel is ready for viewing when the bromophenol blue dye has run completely out of the gel.

### 3.4.2.5 DGGE gel staining and viewing

1. Turn off the power supply and remove gel from tank and holder.
2. Unseal the sides of the glass plates and gently remove one of the glass plates, leaving the gel to rest on the other.

3. Place the gel in a container with approximately 250 ml of 1x TAE containing 0.5 µg/ml ethidium bromide or syber green and stain for about 15 minutes.
4. Place the gel onto a UV transilluminator, examine the gel under UV light (256 nm wavelength) and photograph.
5. Cut out by sterile scalpel the band of interest (strong and well separated).

## 3.5 PCR Amplification of archaeal DNA fragment for clone library

### 3.5.1 Primers and PCR program

DNAs isolated from each part of animal lower digestive tract were diluted 10 times in $ddH_2O$ prior to PCR reactions and 2 µl of the diluted DNA solutions were used as template for PCR reaction. PCR amplification targeting the archaeal 16S rDNA fragment was performed using the specific primer pair Met86F (5´-GCTCAGTAACACGTGG-3´) and Met1340R (5´-CGGTGTGTGCAAGGAG-3´) according to Wright and Pimm (2003). The PCR reaction with primers Met86F and Met1340R was performed using PPP Master Mix kit (Top-Bio, Czech Republic). Each 30 µl PCR mixture contained 75 mMTris-HCl (pH 8.8), 20 mM $(NH4)_2SO_4$, 0.01% Tween 20, 2.5 mM $MgCl_2$, 200 µM dATP, 200 µM dCTP, 200 µM dGTP, 200 µM dTTP, 50 U/ml Taq DNA polymerase, 0.3 µM of each primer and 2 µl of template DNA (10-fold diluted). The following PCR assay was performed: 4 minutes of denaturation step at 94°C, 33 cycles consisting of 1 min at 94°C, 30 seconds at 58°C, 45 seconds at 72°C and final elongation step at 72°C for 2 min (Wright and Pimm, 2003). PCR reaction was carried on the thermocycler Biometra TProfessional (Biometra, Germany). Each PCR product (1.5 - 5 µl) was analysed by agarose gel electrophoresis (0.8%) to evaluate their size and quality and purified using kits as described below. The length of fragment of archaeal 16S rDNA used in this study ("MET" product) was approximately 1200 bp.

### 3.5.2 Agarose gel electrophoresis

The PCR products were checked by agarose gel electrophoresis. Typically 0.8% agarose gels prepared in 1X TBE buffer were used. The agarose was dissolved in the buffer by boiling and poured into plastic trays with a plastic comb fitted to create sample wells and allowed to set.

The gels were run in a Biometra horizontal gel apparatus using 1x TBE as the running buffer. The DNA samples were mixed with loading dye and loaded into the wells. The loading dye aided loading and visualisation of the migration process during electrophoresis. The gels were run at 110 V for 30-40 minutes depending on the gel length. The staining was achieved by the immersion in ethidium bromide staining solution for 15 minutes or the ethidium bromide (0.5 µg/ml final concentration*)* was directly incorporated into the gel. The DNA was visualised on a UV transilluminator (VilberLourmat System, France). 200-1500 kb DNA ladder (Top-Bio, Czech Republic) was run as a molecular size marker in every gel (Fig.3.5 - 1).

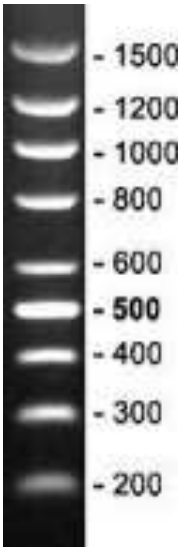


**Figure 3.5– 1. 200-1500 DNA ladder (Top Bio). A representative image of the DNA ladder used for agarose gel electrophoresis in this study.**

## 3.6 Purification and concentration of the PCR product

The fragments amplified by PCR as described above were purified directly from the PCR reaction or from the gel after the agarose gel electrophoresis depending on the numbers of bands. The ´MET´ PCR products from the horse digestive tract were purified by QIAquick Gel Extraction Kit, because during the PCR amplification two bands were generated. The ´MET´ PCR products from the donkey digestive tract were purified by QIAquick PCR Purification Kit, because only one band was generated. The purification procedure is based, in both cases, on the use of a silica membrane assembly that binds the DNA in high-salt buffer followed by elution of the DNA with a low-salt buffer or water. The procedure removes primers, nucleotides, enzymes, salts, agarose, and other impurities from DNA samples. The detailed procedures are described below.

### 3.6.1 Horse: Purification of archaeal 16S rDNA fragment using QIAquick Gel Extraction Kit

The fragments amplified by PCR were purified from the gel after the agarose gel electrophoresis. The band of approximate size 1200 bp was cut from the agarose gel and purified from the gel using QIAquick Gel Extraction kit (Qiagen, Germany) in the following way:

1. The appropriately sized DNA fragment was excised from the agarose gel using a clean scalpel and transferred to a microcentrifuge tube.
2. The size of the gel slice was determined by weight and 3 volumes of Buffer QG were added to a 1 volume of gel.
3. The tube was incubated at 50°C until the gel slice had dissolved completely. To help the gel dissolution, the tube was mixed by vortexing several times during the incubation.
4. After the gel slice had dissolved, 1 gel volume of isopropanol was added to the sample, mixed and incubated for 5 minutes at room temperature.
5. To bind DNA, the sample was applied to a QIAquick spin column and centrifuged at 11000 rpm for 1 minute.
6. The flow-through was discarded.
7. The spin column was washed by adding 750 µl of Buffer PE and centrifuged for 1 minute (11 000 rpm).

8. The flow-through was again discarded and the column was centrifuged for additional 2 minutes.
9. The column was placed in a clean 1.5 ml microcentrifuge tube and the DNA was eluted by adding 80 µl Buffer EB (10 mMTris-Cl. pH 8.5) to the centre of the membrane before it was centrifuged for 2 minutes.

### 3.6.2 Donkey: Purification of archaeal 16S rDNA fragment using QIAquick PCR purification Kit

1. 5 volumes of the Buffer PB were added to 1 volume of the PCR reaction and mixed.
2. To bind DNA, the sample was applied to a QIAquick column and centrifuged at 11 000 rpm for 1 minute.
3. The flow-through was discarded and the QIAquick column is placed back in the same tube.
4. To wash, 750 µl of Buffer PE were added to the QIAquick column and centrifuged for 1 minute.
5. To remove the residual wash buffer, the QIAquick column was centrifuged once more for 2 minutes.
6. The column was placed in a clean 1.5 ml microcentrifuge tube and the DNA was eluted by adding 80 µl Buffer EB (10 mMTris-Cl. pH 8.5) to the centre of the membrane before it was centrifuged for 2 minutes.

### 3.6.3 Concentration of archaeal 16S rDNA fragments

In both the cases, the PCR reaction as performed in a large volume (10 x 30 µl of PCR mixture) and after purification described above the DNA solutions were precipitated with 0.1 volume of 3M Sodium Acetate and 2.5 volume of cold ethanol (98%) and the DNA fragments were precipitated overnight at -20°C. This was follwed by centrifugation at 12 000 rpm for 30 min at 0°C to pellet the archaeal 16S rDNA fragments. The supernatant was sucked off and the sample was air-dried for 20-30 minutes. The DNA precipitate was resuspended in 10 µl of distilled water. The quality and concentration of the fragments from each part of the digestive tract of the two animals was checked using the agarose gel electrophoresis and an appropriate volume (2- 4 µl) was used for the preparation of a clone library.

## 3.7 Cloning library of archaeal 16S rDNA fragments

The cloning reactions and transformations were carried out using **TOPO® TA Cloning® Kit for Sequencing (INVITROGEN)** through chemical transformation using pCR™4-TOPOVectors according to the producer instructions.

### 3.7.1 Cloning of the archaeal 16S rDNA fragment into pCR™4-TOPO vector

The PCR products of approximate length 1200bp were ligated to pCR™4-TOPO (figure 3.7-1). The cloning reaction was set up using the reagents in the order shown in the following scheme:

| Reagent | Volume |
|---|---|
| Fresh PCR product | 2–4 µL |
| Salt Solution | 1 µL |
| Water | add to a total volume of 5 µL |
| TOPO® vector pCR4 (Fig.2) | 1 µL |
| Final Volume | 6 µL |

The microtube containing all the components was mixed gently and incubated at room temperature for 30 minute. After the incubation, the solution was either frozen (-20°C) or 3 µl of solution were directly used for the *Escherichia coli* transformation procedure. The plasmid vector containing the fragment of the archaeal 16S rDNA is thus ready to be introduced into the E. coli cells.

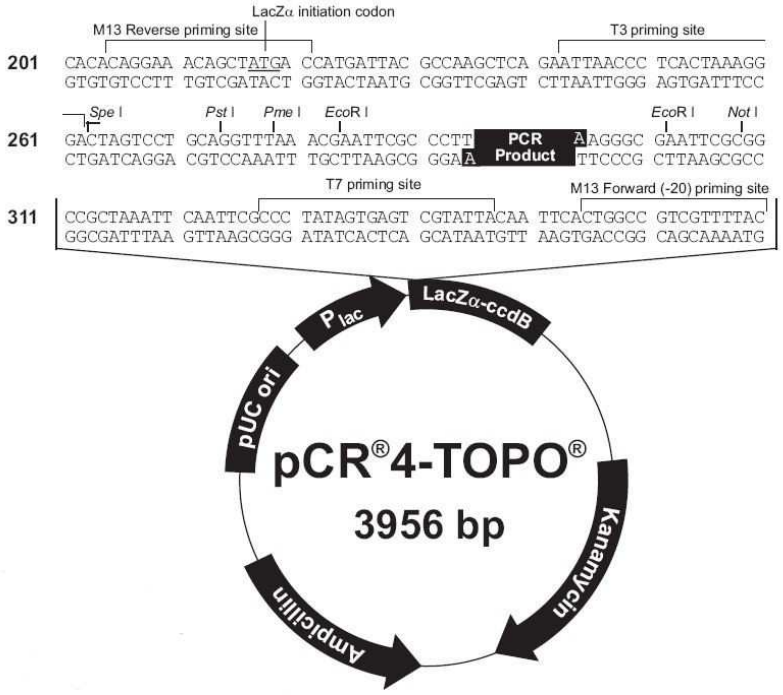


**Figure 3.7- 1.The schematic map of the plasmid vector pCR™4-TOPO.**

### 3.7.2 Transformation of one shot TOP10 competent cells

All the steps in this protocol were carried out as sterile as possible.

1. One tube of *E. Coli* One Shot TOP™10 competent cells for each sample was removed from –80 °C freezer and thawed on ice.
2. 3 µl of the above cloning reaction were added to the tube of the competent cells and mixed gently.
3. The tube was incubated on ice for 30 minutes.
4. The cells were heat-shocked for 45 seconds at 42°C without shaking.
5. The cell tube was transferred back on ice and incubated for 3 minutes
6. 250µl of room temperature S. O. C. medium (see below) were added and the tube was incubated horizontally at 37°C with vigorous shaking at 200 rpm for 2 hours.
7. During this incubation the Luria-Bertani (LB) agar medium containing ampicillin (50µg/ml) was prepared (see below) and poured into Petri dishes.

8. After the incubation, each 50 µl of sample were transferred to the centre of an agar plate containing appropriate antibiotic (together 5 plates) and a sterile spreader was used to spread the solution over the entire surface of the plate.
9. The plate was stored at room temperature until the liquid had been absorbed.
10. The plate was inverted and incubated overnight at 37°C. The success of transformation was determined by the colony.
11. The colonies were picked up using sterilized tooth picks and transferred to the culture tubes containing 1.2 ml of liquid LB medium with 75µg/ml of ampicillin.
12. After overnight incubation at 37°C, 0.5 ml of sterile glycerol was added into each tube and the mixture was stored at -20°C for further analysis or followed with plasmid extraction.

## Reagents and solutions

*S. O. C. medium (pH 7)*
2% Tryptone
0.5% Yeast Extract
10 mM NaCl
2.5 mM KCl
10 mM MgCl2
10 mM MgSO4
20 mM glucose

*LB broth and agar (pH 7)*
1% Tryptone
0.5% Yeast extract
1% Sodium chloride
0.2% Glucose
1.5% Agar (if required)

### 3.7.3 Isolation of plasmid DNA from *E. coli*

The plasmid vector pCR4 containing the archaeal 16S rDNA insert was isolated from *E. coli* cells using the **GenElute™ HP Plasmid Miniprep Kit** (**Sigma-Aldrich**). The kit was used according to the manufacturer's instructions in the following way:

1. 15 ml Falcon tubes filled with 9 mL of LB broth containing the appropriate selective antibiotics (ampicillin: 100 µg/mL) are inoculated with 300 µl of the appropriate sample and incubated at 37°C with vigorous shaking at 200 rpm overnight.
2. On the next day, the cells are harvested with centrifugation at 5000 rpm for 10 minutes and the supernatant is removed. To remove the liquid completely by upsidedown tube onto a piece of paper towel for a few sec.
3. The *E. coli* pellet is resuspended in 200 µL of Resuspension buffer, transferred to 1.5 mL reaction tubes, then 200 µL of Lysis buffer are added and the lysis mix is carefully inverted 5–6 times until the solution is viscous. Then 350 µL of Neutralization buffer are added and again the solution is mixed carefully inverting several times until a white precipitate of genomic DNA and proteins is visible.
4. The white precipitate is pelleted centrifuging at 11000 rpm for 10 minutes.
5. In the meantime a Spin column tube with filter was prepared for the use by the addition of 500 µl of the Column preparation solution and centrifugation at 11000 rpm for 1 minute. The flow-through was discarded.
6. The supernatant was transferred to the Spin column with filter and centrifuged at 11000 rpm for 1 minute to bind the plasmid DNA to the column. The flow through was discarded.
7. 750 µl of Wash solution 2 were added and centrifuged for 1 minute at 11000 rpm. The flow through was discarded and the column was dried by centrifugation at 11000 rpm for 2 minutes.
8. The column was transferred to a fresh tube and 80 µl Elution buffer were added.
9. After last centrifugation (2 minutes at 11 000 rpm) the plasmid DNA was eluted from the column filter and the presence of archaeal 16S rDNA insert inside the plasmid DNA was checked by the restriction enzyme analysis.

### 3.7.4 Restriction enzyme analysis for detection of insert in pCR4 vector

The restriction enzyme digestion was performed to cut out the archaeal 16S rDNA insert from the plasmid. The desired insert inside the plasmid vector is surrounded by the cleavage sites and only the restriction with the enzyme EcoRI splits out the insert from the plasmid. Therefore the enzyme Eco RI (BioLabs – New England) was used for performing the following reaction.

1. The digestion mixture (see below) was incubated overnight at $37^{O}$C.
2. The digestion products were checked on 0.8% agarose gel at 110V for 40min along with the mass ladder. In the gel there were two bands visible, one corresponding to the 16S rDNA PCR product and the second for the plasmid residue. When the PCR product was about 1400 bp in length, the sample was considered as positive.

***Reaction set up***

| | |
|---|---|
| *Eco RI enzyme* | 0.5 µl |
| *10X Eco RI Reaction Buffer* | 1 µl |
| *plasmid DNA isolated* | 9 µl |
| *Sterile distilled water* | 1.5 µl |
| Total | 12 µl |

## 3.8 PCR-RFLP analysis of archaeal 16S rDNA fragments

### 3.8.1 PCR amplification of archaeal 16S rDNA fragments from plasmid DNA

The plasmids with the archaeal 16S rDNA inserts generated in this study as described above in the paragraph 3.7 (Cloning library of the 16S rDNA archaeal fragments) from all the libraries performed for different parts of the horse and of the donkey digestive tract were used as the DNA templates for re-amplification of the 16S rDNA fragments using the primer pair M13F (5'-TGTAAAACGACGGCCAGT-3') and M13R (5'- CAGGAAACAGCTATGACC-3') to generate the material for the RFLP (restriction fragment length polymorphism) analysis. The PCR reaction with primers M13F and M13R was performed using PPP Master Mix Kit (Top-Bio, Czech Republic). Each 30 µl PCR mixture contained 75 mMTris-HCl (pH 8.8), 20 mM $(NH4)_2SO_4$, 0.01% Tween 20, 2.5 mM $MgCl_2$, 200 µM dATP, 200 µM dCTP, 200 µM dGTP, 200 µM dTTP, 50 U/ml Taq DNA polymerase, 0.3 µM of each primer and 1µl of plasmid DNA. The following PCR assay was performed: 5 minutes of denaturation step at 94°C, 30 cycles consisting of 1 min at 94°C, 1 min at 45°C, 1 min at 72°C and final elongation step at 72°C for 5 min.

In order to check the quality and the quantity of the amplification product, 3µl of the each PCR product were subjected to electrophoresis in 0,8% agorose gel at 100 V for 30 min. The PCR products with the expected length ranging from 1000 to 1400 bp were used for the RFLP analysis.

#### 3.8.1.1 In situ prediction of restriction enzymes for RFLP analysis

The Archaeal 16S rDNA from 11 randomly selected positive samples (plasmids with the insert) from horse caecum were re-amplified with M13 primer set and sequenced. The obtained sequences were subjected to computational selection of proper restriction enzymes useful for RFLP analysis. The restriction enzymes were selected using the RestrictionMapper available online (http://www.restrictionmapper.org/) and four different restriction enzymes were considered to be informative because they generated different band patterns of varying sizes and length for distinguishing of tested sequences.

#### 3.8.1.2 Testing of restriction enzymes for RFLP analysis

Sequences of the 11 archaeal 16S rDNA from randomly selected positive samples from horse faeces were subjected to restriction cutting with the following four selected restriction enzymes:

BpmI, MboI, DraIII and MluI. The cut samples were checked using agarose gel and based on their RFLP profile the two restrictases MboI and MluI were selected for RFLP analysis of all the samples.

### 3.8.2 RFLP analysis of archaeal 16S rDNA fragments

The archaeal 16S rDNA PCR products were subjected to the restriction fragment length polymorphism (RFLP) analysis using two different restriction enzymes, Mlu I and Mbo I (Takara Bio). Only one restriction enzyme at a time was applied to the PCR product to avoid confusion and misinterpretation. All the restriction digestions were performed according to the standard procedure provided by the manufacturer. The restriction mixture was performed in a total volume of 12 µl and included: 9 µl PCR-M13 amplified DNA product, 1 µl of restriction buffer (specific for the type of restriction endonuclease), 1.5 µl of sterile Millipure water, 0.5 µl of restriction enzymes (Mlu I or Mbo I). All the digestion reactions were incubated at the optimal temperature (37°C) overnight. The restriction patterns of all the samples from libraries were examined on a horizontal electrophoresis apparatus, using 0.8% agarose gels in 1x TBE buffer. The gels were run at 110 V for 45 min. The fragments sizes were estimated by comparisons with a DNA Marker 200-1500 (Top-Bio). The samples were grouped based on their RFLP patterns, and several representatives of each RFLP profile were subjected to the sequencing (SeqMe,Czech Republic).

## 3.9 Sequencing and Phylogenetic Analysis

The samples exhibiting the same restriction patterns were grouped into the several ribotypes and nucleotide sequences of the different ribotypes were determined by sequencing, using forward primer M13 (5'-TGTAAAACGACGGCCAGT-3'). The obtained sequences (≈ 700 bp) were first edited manually for discarding the ambiguous, skipped bases and for excluding the PCR primer sites, using software Chromas Lite (http://technelysium.com.au/). The full gene sequences were compared automatically using the BLAST against the sequences of methanogen sequences available in the GenBank database (http://www.ncbi.nlm.nih.gov/). The sequences generated in this study and the reference sequences retrieved from NCBI GenBank were converted into FASTA format, aligned and subjected to phylogenetic analysis. The multiple nucleotide sequence alignment was performed using the ClustalW algorithm.

The phylogenetic trees were constructed by UPGMA method (Sneath and Sokal, 1973) and the evolutionary distances were computed by the p-distance method (Nei and Kumar, 2000) using the software program MEGA 5.2 (Molecular Evolutionary Genetics Analysis; Tamura *et al*., 2011) and the trees were evaluated using the bootstrap test based on 1000 replicates. For each part of the horse and of the donkey GIT tract a separate tree was constructed.

### 3.9.1 Reference sequences used in this study

The 16S rRNA archeal gene reference sequences used for the phylogenetic analysis of the methanogens in the hindgut of horse and donkey are listed below.

#### 3.9.1.1 Reference sequences used to construct the phylogenetic trees of the methanogens of the hindgut of horse

Uncultured archaeon PMET35 (AB739387), Uncultured archaeon clone 3D4 (HQ678050), Uncultured archaeon PMET62 (AB739366), Uncultured archaeon HMET24 (AB739326), Methanocorpusculum labreanum (NR074173), Methanobrevibacter sp. NT7 (AJ009959), Methanospirillum hungatei (M60880), Methanofollis liminatans (Y16429), Methanogenium thermophilum (M59129), Methanosarcina barkeri (AY196682), Methanocorpusculum bavaricum (NR042787), Methanimicrococcus blatticola (JQ268013), Methanobrevibacter olleyae (NR043024), Methanobrevibacter ruminantium (NR042784), Methanomassiliicoccus luminyensis (NR118098), Aquifex pyrophilus (NR029172).

#### 3.9.1.2 Reference sequences used to construct the phylogenetic trees of the methanogens of the hindgutof the donkey

Methanobrevibacter ruminantium (AY196666), Methanobrevibacter sp. NT7 (AJ009959), Methanobrevibacter smithii (AY196669), Methanobacterium formicicum (AF169245), Methanimicrococcus blatticola (AY196680), Methanomicrobium mobile (AY196679), Methanospirillum hungatei (AY196683), Methanococcus vannielii (AY196675), Uncultured archaeon clone 3D4 (HQ678050), Uncultured archaeon PMET62 (AB739366), Uncultured archaeon HMET24 (AB739326), Methanocorpusculum labreanum (NR074173), Methanocorpusculum bavaricum (NR042787), Methanomassiliicoccus luminyensis (NR118098), Aquifex pyrophilus (NR 029172).

# 4. RESULTS

## 4.1 DGGE comparison of the archaeal profiles of the different parts of the digestive tract of horse and donkey

The PCR-DGGE study was performed to obtain a preliminary insight and comparison of the diversity of the methanogenic archaea in the samples from six different parts of the horse and donkey digestive tracts. The analyses displayed several band profiles indicating differences in the archaeal community composition among the different parts of the digestive tract. The different intensity of the bands implies the prevalence of relatively low number (2 - 4) of highly represented species indicated by strong band signals.

The figure 4.1-1 shows the DGGE profile of the caecum, four parts of the colon, and faeces of the horse. The band patterns indicate similarity between the right ventral colon (C1) and the left and right dorsal colon (C3 and C4) with one intensive band in the lower part of the gel, and between left ventral colon and faeces (C2 and Fe) with several intensive bands along the gel. The archaeal profile of the caecum (Ce) is different, however, with intensive bands in upper part of the gel, is more closely related to LVC and faeces (C2 and Fe) then to the other three parts of the colon (C1, C3, C4). The DGGE band patterns indicate the lowest methanogenic diversity in the caecum and the highest methanogenic diversity in the faeces and left ventral colon (C2).

The figure 4.1-2 shows the DGGE profile of the caecum, four parts of the colon, and faeces of the donkey. The band patterns indicate similarity, nearly identity, among the left and right dorsal colon (C3, C4), the faeces (Fe) and the caecum (Ce) with two intensive bands in the upper part of the gel and one intensive band in the middle of gel. Similarity (identity) is also apparent for the right and left ventral colon (C1 and C2), which profile is however different from the other parts of the digestive tract with a lower number of bands, indicating a lower methanogenic diversity.

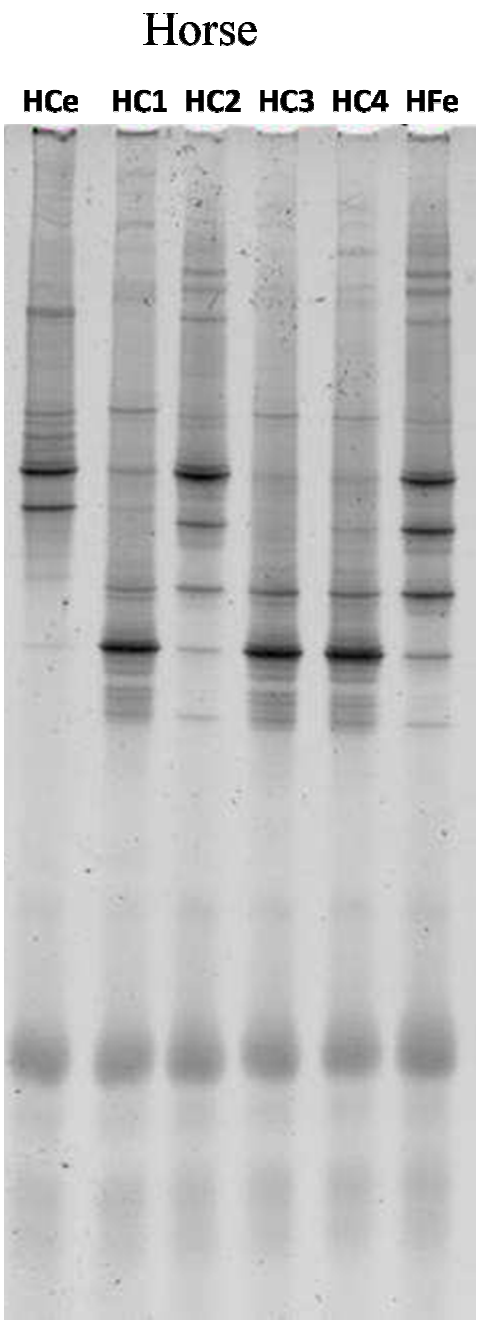


**Figure 4.1-1. DGGE profile of methanogens in the different parts of the horse hindgut. HCe.** caecum; **HC1.** RVC; **HC2.** LVC; **HC3.** LDC; **HC4**. RDC. **HFe**. faeces.

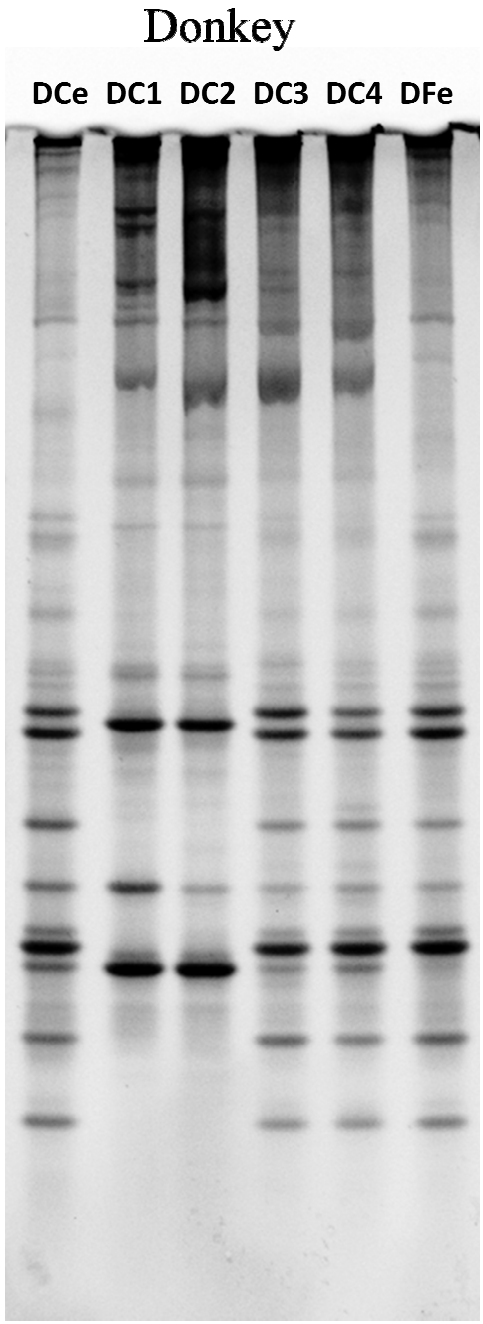


**Figure 4.1-2. DGGE profile of methanogens in the different parts of the donkey hindgut. DCe.** caecum; **DC1.** RVC; **DC2.** LVC; **DC3.** LDC; **DC4**. RDC. **DFe**. faeces.

## 4.2 RFLP analysis of 16S rRNA gene clones of the methanogenic archaea generated from horse and donkey hindgut

A total of 680 clones were generated from twelve archaeal 16S rDNA gene cloning libraries of the different segments of the horse (345 positive clones) and donkey (335 positive clones) hindgut. These clones were differentiated based on the RFLP (restriction fragment length polymorphism) patterns obtained by the splitting of the archaeal 16S rDNA fragments by the restriction enzymes MluI (Figure 4.2-1) and MboI (Figure 4.2-2).

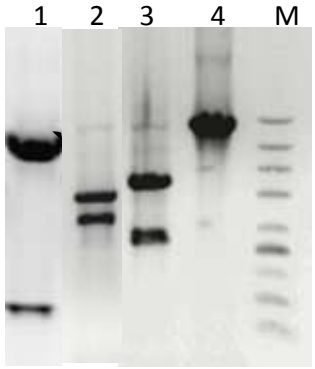


**Figure 4.2-1. Four different RFLP profiles generated by the MluI restriction analysis of the PCR products of the archeal 16S rDNA generated from the content of the different parts of the digestive tract of the horse and donkey. Lane 1: Ribotype 4; Lane 2: Ribotype 3; Lane 3: Ribotype 3; Lane 4: Ribotype 1; Lane M: DNA marker 200–1500 bp.**

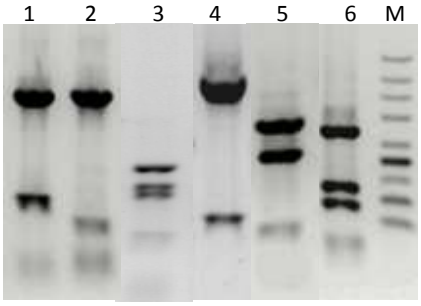


**Figure 4.2-2. Six different most common RFLP profiles generated by the MboI restriction analysis of the PCR products of the archeal 16S rDNA generated from the content of the different parts of the digestive tract of the horse and donkey. Lane 1: Ribotype 3A; Lane 2: Ribotype 3B; Lane 3: Ribotype 1E; Lane 4: Ribotype 1A; Lane 5: Ribotype 1B; Lane 6: Ribotype 2A; Lane M: DNA marker 200–1500 bp.**

Based on the RFLP analysis 23 different ribotypes were detected. Their percentage abundance in the whole hindgut and their distribution in its different parts of the horse and of the donkey are compared in Table 4.2-1. In both the animals the ribotypes 2A, 3A and 3B were dominant, while most of the other ribotypes were less represented, not exceeding more than 1% (10 ribotypes) to 3.5% (5 ribotypes) of total clones. Not all the ribotypes were found in both the animals. Some unshared ribotypes were low numerous, represented by one up to ten clones (≤ 2.9%), such as 1C, 1H, 2N found only in the donkey hingut, and 1D, 1J, 2B, 2C, 2F, 2G, 2I, 3C, 3D, 3E, 3F and 4A found only in the horse hingut. On the other hand, the ribotypes 1A and 1E, spread in the horse hindgut forming together 20%, were not detected at all in the donkey hindgut. In general, the diversity of the ribotypes in the donkey hindgut was much lower. The considerable differences between horse and donkey in incidence of archaeal RFLP ribotypes is graphically expressed in the figures 4.2-3 and 4.2-4.

**Table 4.2-1: RFLP ribotypes and their abundance along the whole hindgut of horse and donkey. Percentage of incidence of the ribotype for each animal is indicated in the brackets.**

| | Horse hindgut | Horse hindgut | Donkey hindgut | Donkey hindgut |
|---|---|---|---|---|
| **RFLP Ribotype** | **N° of positive clones (of total 345 positive clones)(%)** | **Incidence in hindgut segments** | **N° of positive clones (of total 335 positive clones)(%)** | **Incidencein hindgut segments** |
| **1A** | 31 (9%) | HCe, HC1,HC2, HC3, HC4, HFe | 0 | 0 |
| **1B** | 12 (3.5%) | HCe,HC2, HFe | 12 (3.6%) | Ce, C3,Fe |
| **1C** | 0 | 0 | 2 (0.6%) | DFe |
| **1D** | 1 (0.3%) | C2 | 0 | 0 |
| **1E** | 38 (11%) | C3,C4 | 0 | 0 |
| **1F** | 5 (1.4%) | C3,C4 | 4 (1.2%) | C1,C2 |
| **1H** | 0 | 0 | 2 (0.6%) | C2,C4 |
| **1J** | 3 (0.9%) | Ce | 0 | 0 |
| **2A** | 120 (35%) | C1,C2,C3, C4,Fe | 93 (27.8%) | Ce,C1,C2, Fe |
| **2B** | 1 (0.3%) | Fe | 0 | 0 |
| **2C** | 8 (2%) | C2,Fe | 0 | 0 |
| **2D** | 2 (0.6%) | Fe | 1 (0.3%) | C1 |
| **2F** | 3 (0.9%) | C2 | 0 | 0 |
| **2G** | 2 (0.6%) | C4 | 0 | 0 |
| **2I** | 1 (0.3%) | C4 | 0 | 0 |
| **2N** | 0 | 0 | 2 (0.6%) | C2 |
| **3A** | 67 (19.4%) | Ce,C2,C3,Fe | 160 (47.8%) | Ce,C3,C4, Fe |
| **3B** | 27 (7.8%) | C1,C4 | 59 (17.6%) | Ce,C3,C4, Fe |
| **3C** | 10 (2.9%) | C1,C4 | 0 | 0 |
| **3D** | 5 (1.4%) | C2 | 0 | 0 |
| **3E** | 1 (0.3%) | C2 | 0 | 0 |
| **3F** | 1 (0.3%) | C4 | 0 | 0 |
| **4A** | 7 (2%) | Fe | 0 | 0 |

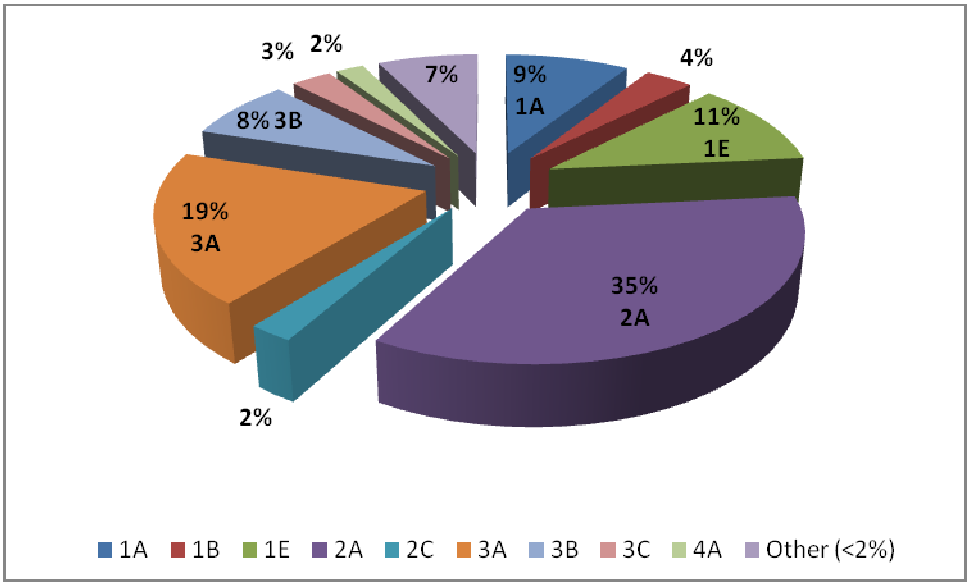


**Figure 4.2-3. Overview of the distribution of the RFLP ribotypes of the methanogens with incidence higher than 2% in the horse hindgut.**

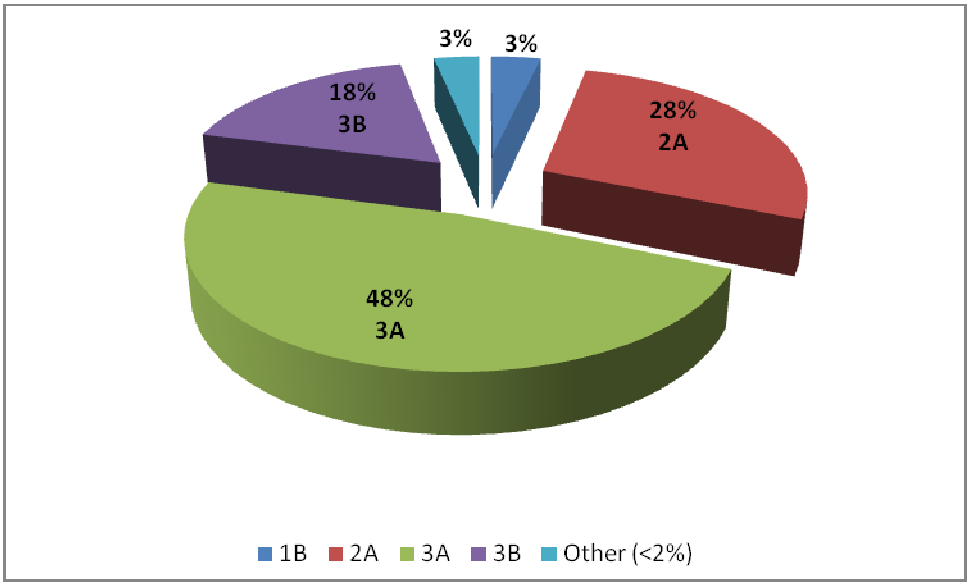


**Figure 4.2-4. Overview of the distribution of the RFLP ribotypes of the methanogens with incidence higher than 2% in the donkey hindgut.**

### 4.2.1 RFLP analysis of the archaeal 16S rRNA gene fragments generated from the horse hindgut

In the horse hindgut, among the 20 different detected ribotypes only the following five were prevalent: the ribotype 2A (35%) followed by the ribotypes 3A (19.4%), 1E (11%), 1A (9%) and 3B (7.8%). Only the ribotype 1A was found in all the studied digestive parts of the horse hindgut. The most numerous ribotype 2A was not present in the caecum. Some ribotypes were detected exclusively in particular segments of the horse hindgut, e.g. the ribotypes 1D, 2F, 3D and 3E were found only in the left ventral colon (HC2); the ribotypes 2G, 2I and 3F in the right dorsal colon (HC4); the ribotypes 2B, 2D, and 4A in the faeces (HFe) and the ribotype 1J in caecum (HCe), all of them with low abundance (≤1.4%). Several clones, representing each RFLP ribotype, were subjected to sequencing with forward primer and compared with the GenBank data. The table 4.2-2 summerizes the incidence, abundance and distribution of the archeal RFLP ribotypes detected in the horse hindgut and for the sequenced fragments the nearest uncultured and/or cultivated related GenBank sequences are specified by GenBank accession number and originator.

**Table 4.2-2. RFLP ribotypes of the archeal 16S rDNA fragments generated from the horse hindgut and similarity values of the sequenced fragments with their nearest relative GenBank sequences.**

| RFLP ribotype | PART OF HORSE GIT: N° of clones (257 sequenced clones) | | | | | |
|---|---|---|---|---|---|---|
| Nearest uncultured relative / Nearest cultured relative | **Hce** | **HC1** | **HC2** | **HC3** | **HC4** | **Hfe** |
| **1A** | 5 (5) | 9 (9) | 3 (2) | 2 (2) | 7(7) | 5 (3) |
| GB acc. No | AB739326 (100%, horse faeces), AB739387 (100%, pony faeces) | AB739326 (100%, horse faeces) | AB739387 (100%,pony faeces) | AB739381 (99%,pony faeces), JX833608 (99%, rhino faeces) | AB739341 (100%, horse faeces) | AB739341 (100%, horse faeces) |
| GB acc. No | Non | Non | Non | Non | *M. blaticola*[1], JQ268013 (98-99%) | *M. blaticola*[1], JQ268013 (98-99%) |
| **1B** | 2 (2) | 0 | 8 (4) | 0 | 0 | 2 (2) |
| GB acc. no | AB739364 (100%, pony faeces) | 0 | DQ402017 (100%, goat rumen), JX833590 (100%, rhino faeces), AB739387 (100%, pony faeces) | 0 | 0 | EU863828 (100%, mule faeces) |
| GB acc. No | Non | 0 | Non | 0 | 0 | Non |
| **1D** | 0 | 0 | 1 (1) | 0 | 0 | 0 |
| GB acc. No | 0 | 0 | JF807068 (100%, cattle rumen) | 0 | 0 | 0 |
| GB acc. No | 0 | 0 | Non | 0 | 0 | 0 |
| **1E** | 0 | 0 | 0 | 29 (15) | 9 (4) | 0 |
| GB acc. No | 0 | 0 | 0 | AB739326 (100%,horse faeces) | AB739326 (100%, horse faeces) | 0 |
| GB acc. No | 0 | 0 | 0 | Non | Non | 0 |
| **1F** | 0 | 0 | 0 | 4 (3) | 1 (1) | 0 |
| GB acc. No | 0 | 0 | 0 | AB739366 (85-100%, pony faeces) | KC454162 (100%, sika deer) | 0 |
| GB acc. No | 0 | 0 | 0 | Non | Non | 0 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **1J** | 3 (3) | 0 | 0 | 0 | 0 | 0 |
| GB acc. No | FJ155596 (86%,saline lakes) | 0 | 0 | 0 | 0 | 0 |
| GB acc. No | *M.labreanum*[2] NR074173 (86%) | 0 | 0 | 0 | 0 | 0 |
| **2A** | 0 | 13(13) | 33 (14) | 13 (13) | 29 (14) | 32 (23) |
| GB acc. No | 0 | AB739366 (100%, pony faeces), AB739357 (99%, pony faeces) | AB739366 (100%, pony faeces) | AB739366 (100%, pony faeces), AB739324 (100%, horse faeces) | AB739324 (100%, horse faeces), AB739366 (100%, pony faeces), AB739369 (98%, pony faeces) | AB739366 (99- 100%, pony faeces) |
| GB acc. No | 0 | *Methanobrevi bacter sp*. AJ009959 (100%) | *Methanobrevi bacter sp.* AJ009959 *(*100%), KF697721 (100%) | *Methanobrevi bacter sp*. KF697721 (100%), AJ009959 (100%) | Non | Non |
| **2B** | 0 | 0 | 0 | 0 | 0 | 1 (1) |
| GB acc. No | 0 | 0 | 0 | 0 | 0 | JQ179646 (100% ,cow rumen) |
| GB acc. No | 0 | 0 | 0 | 0 | 0 | Non |
| **2C** | 0 | 0 | 5 (4) | 0 | | 3 (3) |
| GB acc. no | 0 | 0 | AB 739366 (100%, pony faeces) , AB906082 (100%, buffalo rumen) | 0 | | JF682979 (100%, cow rumen) |
| GB acc. No | 0 | 0 | Non | 0 | | *Methanobrevi bacter sp.* KF697721 (100%) |
| **2D** | 0 | 0 | | 0 | 0 | 2 (1) |
| GB acc. No | 0 | 0 | | 0 | 0 | AB739350 (100%, horse faeces) |
| GB acc. No | 0 | 0 | | 0 | 0 | Non |
| **2F** | 0 | 0 | 3 (2) | 0 | 0 | 0 |
| GB acc. No | 0 | 0 | HM038367 (86-87%,cattle rumen) | 0 | 0 | 0 |
| GB acc. No | 0 | 0 | Non | 0 | 0 | 0 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **2G** | 0 | 0 | 0 | 0 | 2 (2) | 0 |
| GB acc. No | 0 | 0 | 0 | 0 | EU413658 (100%, reindeer rumen) | 0 |
| GB acc. No | 0 | 0 | 0 | 0 | 0 | 0 |
| **2I** | 0 | 0 | 0 | 0 | 1 (1) | 0 |
| GB acc. No | 0 | 0 | | 0 | AB739342 (100%, horse faeces) | 0 |
| GB acc. no | 0 | 0 | | 0 | *M. blaticola*[1] JQ268013 (99%) | 0 |
| **3A** | 45 (45) | 0 | 7 (6) | 6 (6) | 0 | 9 (7) |
| GB acc. no | GU391231 (99-100%, pig caecum), HQ678050 (96-97%, bioreactor) | 0 | HQ678050 (96-100% , bioreactor) | AB 739381 (100%, pony faeces), AB739384 (100% ,pony faeces) | 0 | HQ678050 (99% , bioreactor), AB739344 (100%, horse faeces), KC841482 (100%, tadpole gut) |
| GB acc. no | *M. labreanum*[2] NR074173-CP000559 (100%), *Methanocorpusculum sp*. AY260434-AB288279 (97-100%) | 0 | *M. labreanum*[2] NR074173 ( 99-100%), *M. sinense*[4] NR104804 (100%), *Methanocorpusculum sp.* AY260434 (96-100%) | Non | 0 | *M. bavaricum*[3] NR042787 (99%), *Methanocorpusculum sp*. AY260434 (98-99%) |
| **3B** | 0 | 11 (9) | 0 | 0 | 16 (8) | 0 |
| GB acc. no | 0 | AB739381-AB739380-AB739382-AB739385 (100%,pony faeces), AB739351 (100%, horse faeces) | 0 | | AB739381 (100%, pony faeces) | 0 |
| GB acc. no | 0 | Non | 0 | Non | Non | 0 |
| **3C** | 0 | 8 (6) | 0 | 0 | 2 (2) | 0 |
| GB acc. no | 0 | AB739344 (100%, horse faeces) | 0 | 0 | AB739381 (100%, pony faeces) | 0 |

| | | | | | | |
|---|---|---|---|---|---|---|
| GB acc. no | 0 | Non | 0 | 0 | Non | 0 |
| **3D** | 0 | 0 | 5 (5) | 0 | 0 | 0 |
| GB acc. no | 0 | 0 | FJ155596 (98%, saline lakes), AB739379 (100% ,pony faeces) | 0 | 0 | 0 |
| GB acc. no | 0 | 0 | M. labreanum[2] NR074173 (100%) | 0 | 0 | 0 |
| **3E** | 0 | 0 | 1(1) | 0 | 0 | 0 |
| GB acc. no | 0 | 0 | AB739382 (93%,Pony faeces ) | 0 | 0 | 0 |
| GB acc. no | 0 | 0 | *M.labreanum*[2] NR029086 (93%) | 0 | 0 | 0 |
| **3F** | 0 | 0 | 0 | 0 | 1 (1) | 0 |
| GB acc. no | 0 | 0 | 0 | 0 | AB739341 (100%, horse faeces) | 0 |
| GB acc. no | 0 | 0 | 0 | 0 | *M. blaticola*[1] JQ268013 (99%) | 0 |
| **4A** | 0 | 0 | 0 | 0 | 0 | 7 (7) |
| GB acc. no | 0 | 0 | 0 | 0 | 0 | JF807279-JF807268-JF807269 (100%, yak rumen) |
| GB acc. no | 0 | 0 | 0 | 0 | 0 | Non |

[1]***Methanomicroccocus blaticola***
[2]***Methanocorpusculum labreanum***
[3]***Methanocorpusculum bavaricum,***
[4]***Methanocorpusculum sinense***

### 4.2.2 RFLP analysis of the archaeal 16S rRNA gene fragments generated from the donkey hindgut

In the donkey hindgut, among the 9 different detected ribotypes only three were highly prevalent: the ribotype 3A (47.8%) followed by the ribotypes 2A (27.8%) and 3B (17.6%). No ribotype was found in all the studied parts of the digestive tract, however the most numerous ribotypes 3A and 3B were widespread, but not detected in the right and left ventral colon (DC1, DC2), which were alternatively populated by the ribotype 2A. Some ribotypes were detected exclusively in particular segments of the donkey hindgut, e.g. the ribotype 2D was found only in the right ventral colon (DC1), the ribotype 2N in the left ventral colon (DC2), the ribotype 1C in the faeces (DFe), all of them with abundance $\leq 0.6\%$. Tha table 4.2-3 summerizes the incidence, abundance and distribution of the archeal RFLP ribotypes detected in the donkey hindgut and for the sequenced fragments the nearest uncultured and/or cultivated related GenBank sequences are specified by the GenBank accession number and originator.

**Table 4.2-3. RFLP ribotypes of the archeal 16S rDNA fragments generated from the donkey hindgut and similarity values of the sequenced fragments with their nearest relative GenBank sequences.**

| RFLP ribotype | PARTS OF DONKEY GIT: No of clones (165 sequenced clones) | | | | | |
|---|---|---|---|---|---|---|
| Nearest uncultured relative / Nearest cultured relative | Dce | DC1 | DC2 | DC3 | DC4 | DFe |
| **1B** | 4(4) | 0 | 0 | 5 (5) | 0 | 3(3) |
| GB acc. no | KM650110 (99-100%, takin rumen), | 0 | 0 | JX522655 (100%, pig colon), HQ616013 (100%, cattle rumen), KM650110 (96%, takin rumen) | 0 | KM650110 (100%, takin rumen), JX522655 (100%, pig colon) |
| GB acc. no | Non | 0 | 0 | Non | 0 | Non |
| **1C** | 0 | 0 | 0 | 0 | 0 | 2 (2) |
| GB acc. no | | | | | | AB739382 (96-99%, pony faeces) |
| GB acc. no | | | | | | |
| **1F** | 0 | 1 (1) | 3 (3) | 0 | 0 | 0 |
| GB acc. no | | EU413598 (100%, rein deer) | JF951780 (99%-100%, buffalo rumen) | 0 | 0 | 0 |
| GB acc. no | 0 | Non | Non | 0 | 0 | 0 |
| **1H** | 0 | 0 | 1 (1) | 0 | 1 (1) | 0 |
| GB acc. no | 0 | 0 | JQ179712 (100%, cattle rumen) | 0 | AY464786 (99%, cow rumen) | 0 |
| GB acc. no | 0 | 0 | *M. mobile*[4] AY196679 (100%) | 0 | *M. mobile*[4] AY196679 (99%) | 0 |
| **2A** | 2 (2) | 41 (23) | 49 (21) | 0 | 0 | 1 (1) |
| GB acc. no | JF951780 (100%, buffalo rumen) | AB739366 (100%, pony faeces), JF951780 (100%, buffalo rumen) | AB739366 (100%, pony faeces) | 0 | 0 | JN192495 (97%, orangutan colon) |
| GB acc. no | *M. ruminantium*[5] | *M. ruminantium*[5] | *M. ruminantium*[5] | 0 | 0 | *Methano bacterium sp.* |

| | | | | | | |
|---|---|---|---|---|---|---|
| | KP123415 (100%) | KP123415 (100%), *M. ruminantium*[5] KP123400 (100%) | KP123415 (100%) | | | DQ677518 (96%) |
| **2D** | 0 | 1 (1) | 0 | 0 | 0 | 0 |
| GB acc. no | 0 | EU413638 (100%, rein deer) | 0 | 0 | 0 | 0 |
| GB acc. no | 0 | Non | 0 | 0 | 0 | 0 |
| **2N** | 0 | 0 | 2 (2) | 0 | 0 | 0 |
| GB acc. no | 0 | 0 | KC454261 (100%, sika deer rumen) | 0 | 0 | 0 |
| GB acc. no | 0 | 0 | Non | 0 | 0 | 0 |
| **3A** | 16 (10) | 0 | 0 | 38 (16) | 47 (15) | 59 (14) |
| GB acc. no | HQ678050 (99%, bioreactor), GU391231 (100%, pig caecum) | 0 | 0 | GU391231 (100% pig caecum), HQ678050 (99%, bioreactor) | GU391231 (100%, pig caecum), HQ678050 (99%, bioreactor) | GU391231 (100%, pig caecum), HQ678050 (100%, bioreactor) |
| GB acc. no | *Methanocorpusculum sp*. AY260434 (99%), *M. labreanum* NR074173 (99%) | 0 | 0 | *M.labreanum*[2] NR029086 (99%), *Methanocorpusculum* sp. AY260434 (99%) | *M. labreanum*[2] NR029086 (99%), *Methanocorpusculum sp*. AY260434 (99%) | *M.labreanum*[2] NR074173 (100%), *Methanocorpusculum sp.* AY260434 (100%) |
| **3B** | 9 (9) | 0 | 0 | 12 (10) | 11 (10) | 27 (12) |
| GB acc. no | AB739395-AB739400-AB739401 (100%, pony faeces), HQ678050 (99%, bioreactor) | 0 | 0 | HQ678050 (99%, bioreactor) | AB739401 (100%,pony faeces), HQ678050 (99%, bioreactor) | AB739381-AB739384-AB739394-AB739401 (100%, pony faeces), HQ678050 (99%, bioreactor) |
| GB acc. no | *Methanocorpusculum sp.* AY260434 (99%) | 0 | 0 | *Methanocorpusculum sp.* AY260434 (99%) | *Methanocorpusculum sp.* AY260434 (99%) | *Methanocorpusculum sp.* AY260434 (99%) |

[1] ***Methanomicroccocus blaticola***

[2] ***Methanocorpusculum labreanum***

[3] ***Methanocorpusculum bavaricum***

[4] ***Methanomicrobium mobile***

[5] ***Methanobrevibacter ruminantiun***

## 4.3 Phylogenetic analysis of the archaeal 16S rRNA sequences of the horse hindgut

### 4.3.1 Diversity of the archaeal 16S rDNA sequences generated from the horse caecum

A total of 55 positive clones were generated from the archaeal 16S rDNA gene cloning library of the horse caecum (HCe). These clones were differentiated based on the RFLP patterns and distributed into 4 ribotypes as reported in the following scheme.

| Number of clones | Incidence | Ribotype of belonging |
|---|---|---|
| **45** | 82% | 3A |
| **5** | 9% | 1A |
| **3** | 5% | 1J |
| **2** | 4% | 1B |

All these 55 positive clones were sequenced with forward primer, the highest similarity of these sequences with the GenBank database entries was assessed and the phylogenetic analysis was performed.

The sequences of the ribotype 3A were highly similar to the uncultured archeons obtained from the pig caecum (99-100% sequence similarity with the entry no. GU391232) and from the anaerobic bioreactor (96-100% sequence similarity with the entry no. HQ678050) and from the cultured methanogenss *Methanocorpusculum labreanum* (99-100% sequence similarity with the entries nos. NR074173 and CP000559) and *Methanocorpusculum sp.* (97-100% sequence similarity with the entries nos. AY260434 and AB288279). The sequences of the ribotype 1A were highly similar to the uncultured archeons obtained from the horse faeces (100% sequence similarity with the entry no. AB739326) and from the pony faeces (100% sequence similarity with the entry no. AB739387). No similarity with the cultured methanogens was found. The sequences of the ribotype 1J exhibited a low similarity with the uncultured archeons from saline lake (86% sequence similarity with the entry no. FJ155596) and also a low similarity with the cultured methanogen *Methanocorpusculum sp.* (86% sequence similarity with the entry no. NR074173). The sequences of the ribotype 1B were highly similar to the uncultured archeons obtained from the pony faeces (100% sequence similarity with the entry no. AB739364).

The phylogenetic analysis of all these 55 archaeal 16S rRNA gene sequences revealed that the methanogens of the horse caecum were divided into two main clusters (Figure 4.3-1). The first largest cluster grouped with the order Methanomicrobiales and contained the sequences of the ribotype 3A. The uncultured sequences generated in this study formed two subclusters and between them the subclade of the *Methanocorpusculum* strains was embedded. The subclusters were supported by high bootstrap values. The second cluster grouped with the Methanomassiliicoccales and was devided into three subclades, which did not correspond strictly with the ribotypes, but contained mixed sequences of the ribotypes 1A/1B/1J. This grouping was not supported by high bootstrap values. The cultured strain *Methanomassiliicoccus luminyensis* formed the sister cluster of these three subclades.

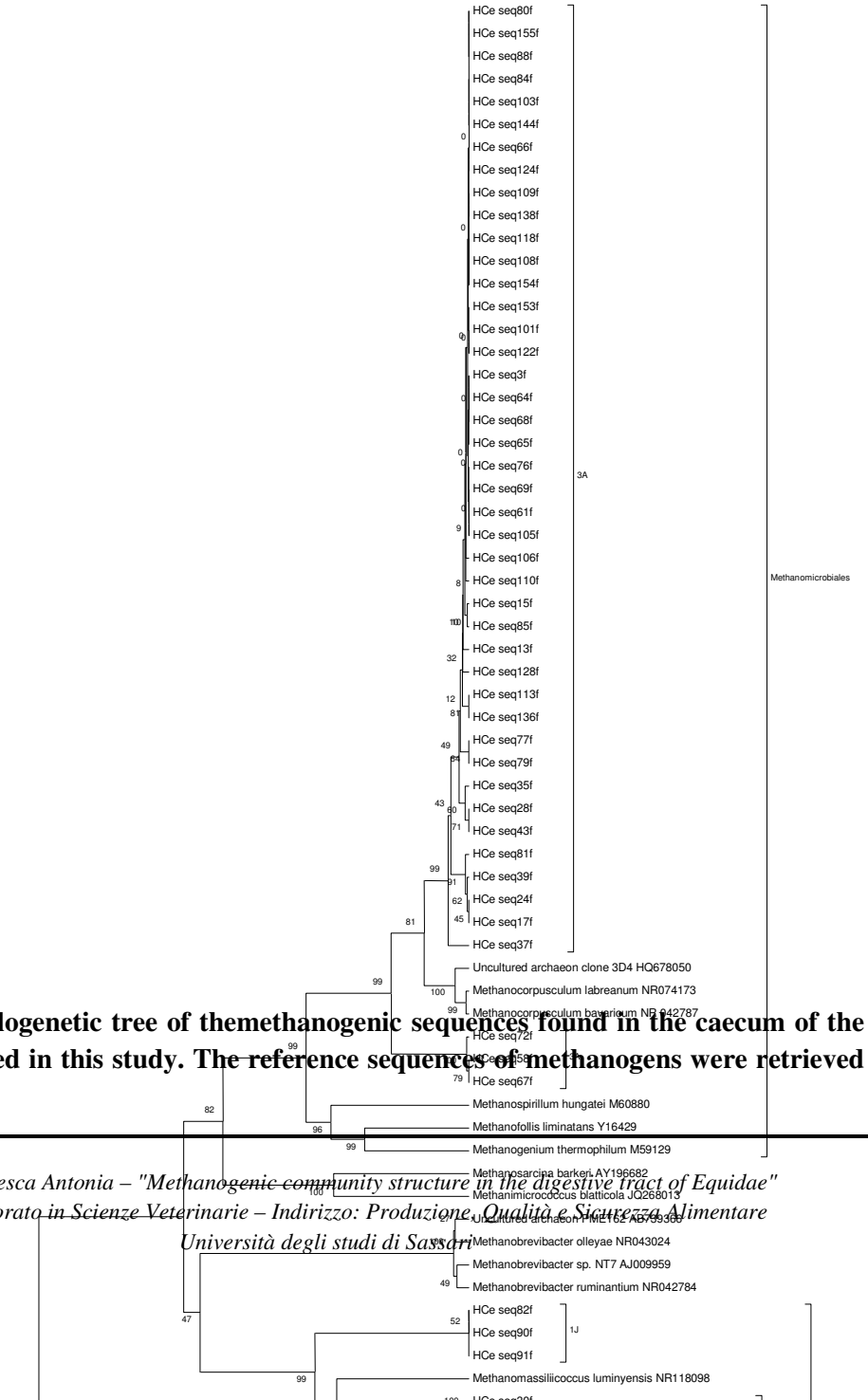


**Figure 4.3-1. Phylogenetic tree of themethanogenic sequences found in the caecum of the horse and analyzed in this study. The reference sequences of methanogens were retrieved**

**from the GenBank and phylogenetically compared with the 55 sequences generated in this study. The Aquifex pyrophilus sequence was used as an outgroup to root the tree. The evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973). The optimal tree with the sum of the branch length = 1.24574769 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with the branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000) and are in the units of the number of base differences per site. The analysis involved 71 nucleotide sequences. All the ambiguous positions were removed for each sequence pair. There were a total of 1609 positions in the final dataset. The evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011).**

### 4.3.2 Diversity of the archaeal 16S rDNA sequences generated from the horse right ventral colon

A total of 41 positive clones were generated from the archaeal 16S rDNA gene cloning library of the horse right ventral colon (HC1). These clones were differentiated based on the RFLP patterns and distributed into 4 ribotypes as reported in the following scheme.

| Number of clones | incidence | Ribotype of belonging |
|---|---|---|
| **13** | 32% | 2A |
| **11** | 27% | 3B |
| **9** | 22% | 1A |
| **8** | 19% | 3C |

37 positive clones were sequenced with forward primer, the highest similarity of these sequences with the GenBank database entries was assessed and the phylogenetic analysis was performed. The sequences of the ribotype 2A were highly similar to the uncultured archeons obtained from the pony faeces (100% sequence similarity with the entry no. AB739366, 99% sequence similarity with the entry no. AB739357) and the cultured methanogen *Methanobrevibacter* sp. (100% sequence similarity with the entry no. AJ009959). The sequences of the ribotype 1A were highly similar to the uncultured archeon obtained from the horse faeces (100% sequence similarity with the entry no. AB739326). The sequences of the ribotype 3C were highly similar to the uncultured archeon obtained from the horse faeces (100% sequence similarity with the entry no. AB739344). The sequences of the ribotype 3B were highly similar to the uncultured archeons obtained from pony faeces (100% sequence similarities with the entries nos. AB 739380,-81,-82,-85) and horse faeces (100% sequence similarity with the entry no. AB739351).

The phylogenetic analysis of all the 37 archaeal 16S rRNA gene sequences revealed that the methanogens of the horse right ventral colon were divided into three main clusters (Figure 4.3-2). The first cluster contained sequences of the ribotype 2A and grouped with the Methanobacteriales. This grouping was supported by high bootstrap values. Inside this cluster the sequences generated in this study formed sister cluster of cultured *Methanobrevibacter* strains, only one sequence (HC1 seq2f) clustered separately. The second cluster contained sequences of the ribotype 1A and grouped with the Methanomassiliicoccales. This grouping was

also supported by high bootstrap values. Inside this cluster two sequences (HC1 seq60f and seq62f) fomed subcluster and together with other uncultured sequences generated in this study formed a sister cluster of cultured strain of *Methanomassiliicoccus luminyensis*. The third main cluster grouping with the Methanomicrobiales was divided into several subclusters. Both sister subclusters of here generated uncultured sequences of the ribotype 3C and 3B formed a sister cluster of the cultured strains of *Methanocorpusculum* and one sequence (HC1 seq3f) represented the adjacent cluster.

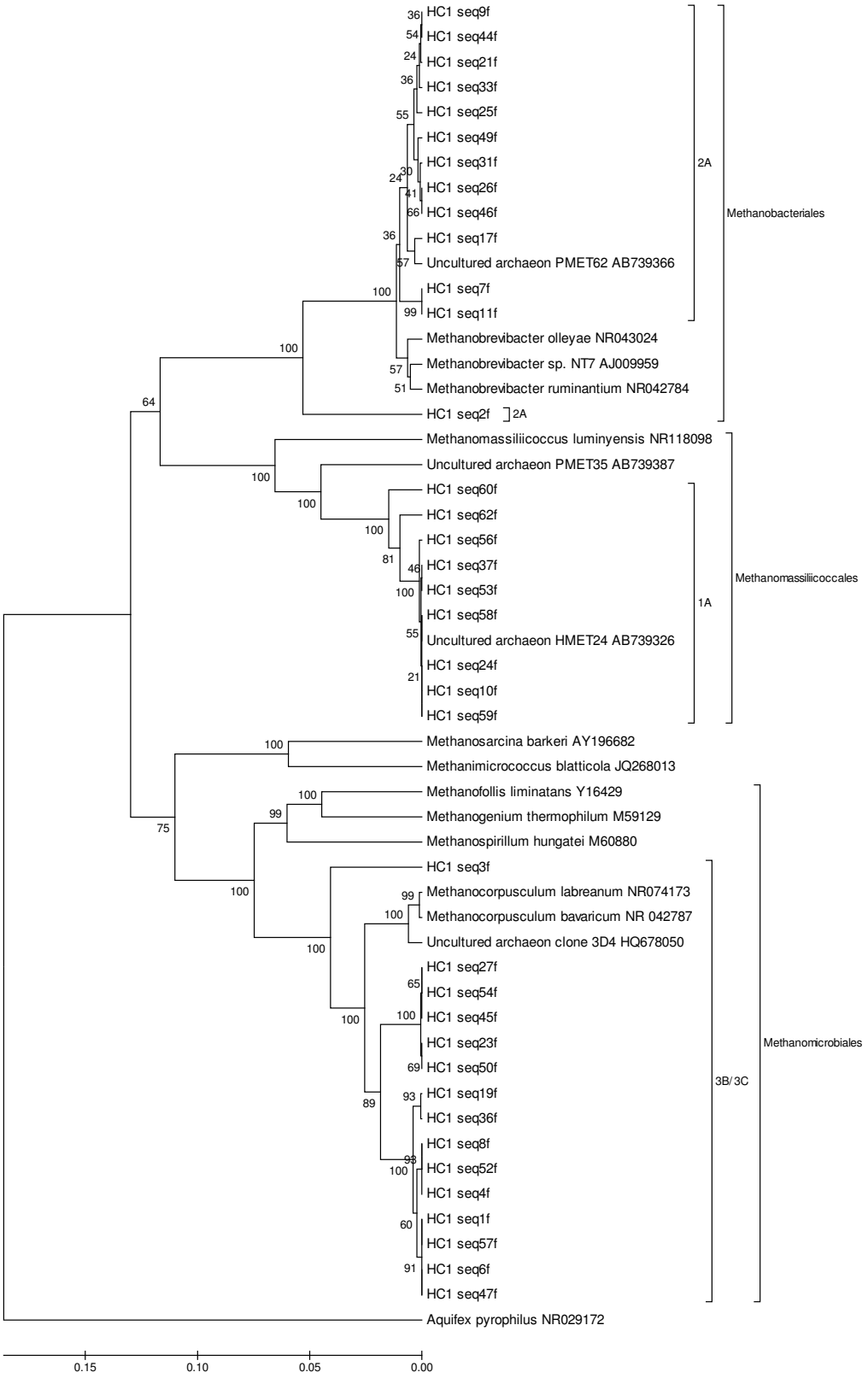


**Figure 4.3-2.** The phylogenetic tree description is reported in the following page.

**Figure 4.3-2. Phylogenetic tree of the methanogenic sequences found in the right ventral colon of the horse and analyzed in this study. The reference sequences of methanogens were retrieved from the GenBank and phylogenetically compared with the 37 sequences generated in this study. The Aquifex pyrophilus sequence was used as an outgroup to root the tree. The evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973). The optimal tree with the sum of the branch length = 1.30952862 is shown. The percentage of the replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with the branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000) and are in the units of the number of base differences per site. The analysis involved 53 nucleotide sequences. All the ambiguous positions were removed for each sequence pair. There were a total of 1613 positions in the final dataset. The evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011).**

### 4.3.3 Diversity of the archaeal 16S rDNA sequences generated from the horse left ventral colon

A total of 67 positive clones were generated from the archaeal 16S rDNA gene cloning library of the horse left ventral colon (HC2). These clones were differentiated based on the RFLP patterns and distributed into 9 ribotypes as reported in the following scheme.

| Number of clones | incidence | Ribotype of belonging |
|---|---|---|
| **34** | 51% | 2A |
| **8** | 12% | 1B |
| **7** | 10.5% | 3A |
| **5** | 7.5% | 2C |
| **5** | 7.5% | 3D |
| **3** | 4.5% | 1A |
| **3** | 4.5% | 2F |
| **1** | 1.5% | 1D |
| **1** | 1.5% | 3E |

39 positive clones were sequenced with the forward primer, the highest similarity of these sequences with the GenBank database entries was assessed and the phylogenetic analysis was performed. The sequences of the ribotypes 2A were highly similar to the uncultured archeons obtained from the pony faeces (100% sequence similarity with the entry no.AB 739366) and the cultured methanogen *Methanobrevibacter sp.* (100% sequence similarity with the entries nos. AJ009959 and KF697721). The sequences of the ribotype 1B were highly similar to the uncultured archeons obtained from the goat rumen (100% sequence similarity with the entry no. DQ402017), rhino faeces (100% sequence similarity with the entry no. JX833590) and pony faeces (100% sequence similarity with the entry no. AB739387). The sequences of the ribotype 3A showed a high similarity both with the uncultured archeon obtained from the anaerobic bioreactor (96-100% sequence similarity with the entry no. HQ678050) and the cultured species of *Methanocorpusculum*: *Methanocorpusculum* sp. (100% sequence similarity with the entry no. AY260434), *Methanocorpusculum sinense* (100% sequence similarity with the entry no. NR104804) and *Methanocorpusculum labreanum* (100% sequence similarity with the entry no. NR074173). The sequences of the ribotype 2C were highly similar to the uncultured archeons

obtained from the pony faeces (100% sequences similarity with the entry no. AB739366) and the buffalo rumen (100% sequences similarity with the entry no.AB906082). The sequences of the ribotype 3D were highly similar to the uncultured archeons obtained from the saline lake (98% sequences similarity with the entry no. FJ155596) and the pony faeces (100% sequence similarity with the entry no. AB739379) and the cultured methanogen *Methanocorpusculum labreanum* (100% sequence similarity with the entry no. NR074173). The sequences of the ribotype 1A were highly similar to the uncultured archeon obtained from the pony faeces (100% sequence similarity with the entry no. AB739387). The sequences of the ribotype 2F exhibeted a low similarity with the uncultured archeon from the cattle rumen (86-87% sequence similarity with the entry no. HM038367). The only one sequence of the ribotype 1D was highly similar to the uncultured archeon from the cattle rumen (100% sequence similarity with the entry no. JF807068). The only one sequence of the ribotype 3E exhibited low similarity with the uncultured archeon from the pony faeces (93% sequencesimilarity with the entry no. AB739382) and the cultured *Methanocorpusculum labreanum* (93% sequence similarity with the entry no. NR029086).

The phylogenetic analysis of 39 archaeal 16S rRNA gene sequences revealed that the methanogens of the horse left ventral colon were divided into three main clusters (Figure 4.3-3). The first main cluster grouped with the Methanobacteriales and contained the sequences of the ribotypes 2A and 2C closely related to the genus *Methanobrevibacter*. Two sequences (HC2 seq13f and HC2 seq34f) clustered separately, however without a good support cluster. The second cluster grouped with the Methanomicrobiales and the sequences generated in this study formed two not well supported subclusters. One subcluster containing the sequences of the ribotype 3D formed sister cluster of the *Methanocorpusculum* strains. The second subcluster contained a mixture of sequences of the ribotypes 3A and 3D. The single unknown sequence HC2 seq37 (3E) clustered separately and was embedded between the uncultured sequences related to Methanocorpusculaceae and the cluster of the cultured strains of Methanomicrobiaceae and Methanospirillaceae. The third well supported cluster grouping with the Methanomassiliicoccales was devided into several small subclades and contained the mixture of the sequences of ribotypes 1A/1B/1D. Two sequences of the uncultured archeon of the ribotype 2F (HC2 seq63f and HC2 seq75f) clustered separately forming well supported sister group of Methanomassiliicoccales.

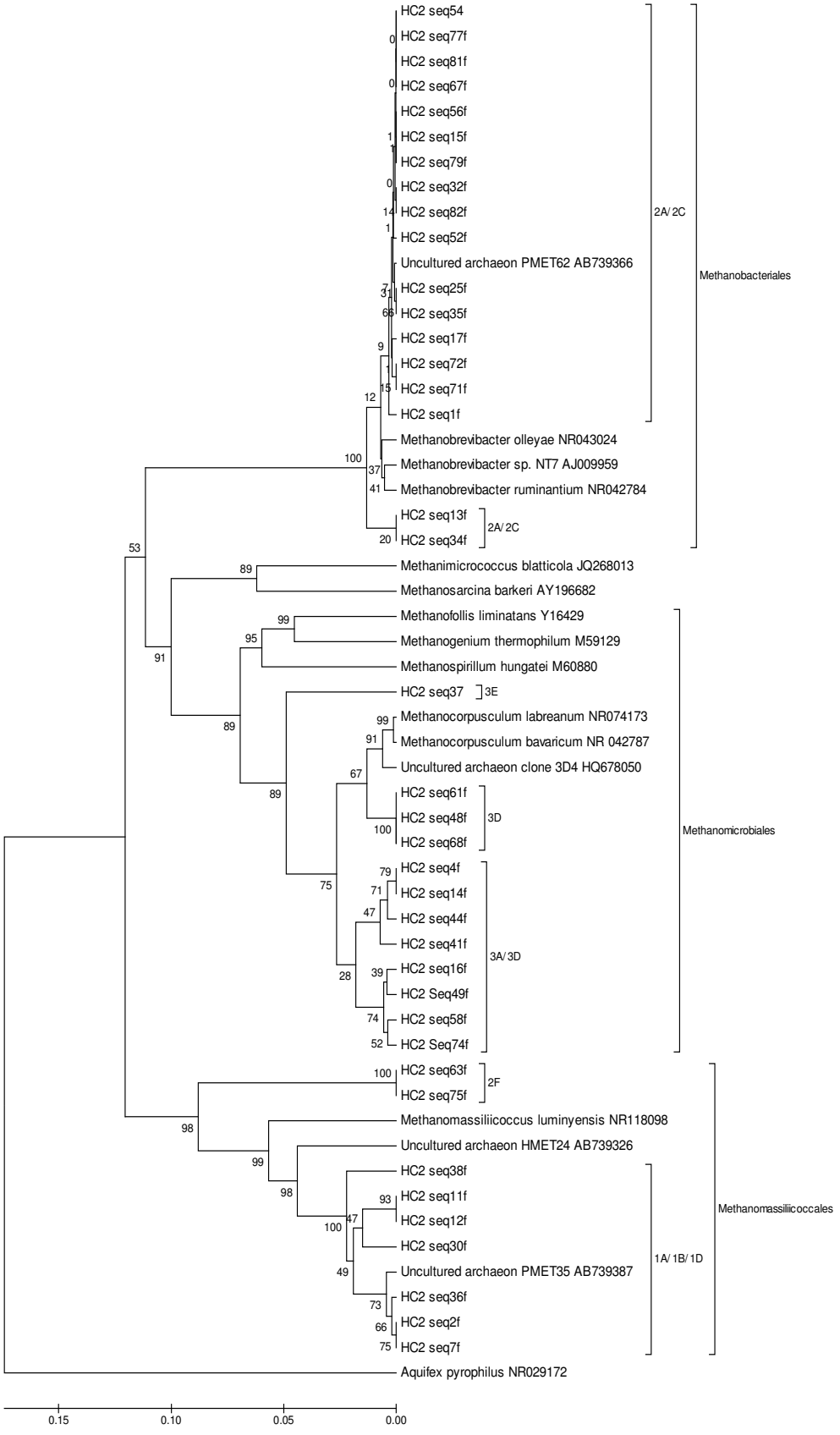


**Figure 4.3-3.** The phylogenetic tree description is reported in the following page**.**

**Figure 4.3-3. Phylogenetic tree of the methanogenic sequences found in the left ventral colon of the horse and analyzed in this study. The reference sequences of methanogens were retrieved from the GenBank and phylogenetically compared with the 39 sequences generated in this study. The Aquifex pyrophilus sequence was used as an outgroup to root the tree. The evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973). The optimal tree with the sum of branch length = 1.34729643 is shown. The percentage of the replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with the branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000) and are in the units of the number of base differences per site. The analysis involved 55 nucleotide sequences. All the ambiguous positions were removed for each sequence pair. There were a total of 1622 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011).**

### 4.3.4 Diversity of the archaeal 16S rDNA sequences generated from the horse left dorsal colon

A total of 54 positive clones were generated from the archaeal 16S rDNA gene cloning library of the horse left dorsal colon (HC3). These clones were differentiated based on the RFLP patterns and distributed into 5 ribotypes as reported in the following scheme.

| Number of clones | incidence | Ribotype of belonging |
|---|---|---|
| **29** | 54% | 1E |
| **13** | 24% | 2A |
| **6** | 11% | 3A |
| **4** | 7% | 1F |
| **2** | 4% | 1A |

39 positive clones were sequenced with the forward primer, the highest similarity of these sequences with the GenBank database entries was assessed and the phylogenetic analysis was performed. The sequences of the ribotype 1E were highly similar to the uncultured archeons obtained from the pony faeces (100% sequence similarity with the entry no. AB739326) and only one clone (HC3 seq14f) exhibited the 100% sequence similarity with the uncultured archeon from the yak rumen (entry no. JF807264). The sequences of the ribotype 2A were highly similar to the uncultured archeons obtained from the pony faeces (100% sequence similarity with the entry no.AB739366) and the horse faeces (100% sequence similarity with the entry no.AB739324) and the cultured methanogen *Methanobrevibacter* sp. (100% sequence similarity with the entries nos. AJ009959 and KF697721). The sequences of the ribotype 3A were highly similar to the uncultured archeons from the pony faeces (100% sequence similarity with the entries nos. AB739381 and AB739384). The sequences of the ribotype 1A were highly similar to the uncultured archeons obtained from the pony faeces (99% sequence similarity with the entry no. AB739381) and the rhino faeces (99% sequence similarity with the entry no. JX833608). The sequences of the ribotype 1F exhibited a different range of similarity to the uncultured archeon from the pony faeces AB739366 (HC3 seq11f exhibited only low 85% similarity, HC3 seq9f exhibited 96% similarity and HC3 seq8f exhibited 100% similarity).

The phylogenetic analysis of all 39 archeal 16S rRNA gene sequences revealed that the methanogens of the horse left dorsal colon were divided into four main clusters (Figure 4.3-4).

The first cluster grouped with the Methanobacteriales and was devided into two subclusters. The biggest subcluster contained all the sequences of the ribotype 2A, which were related to the species of the genus *Methanobrevibacter*. A smaller subcluster contained unknown sequences of the ribotype 1F. The subclusters however were not well supported. The second cluster containing the sequences of the ribotype 1E grouped with the Methanomassiliicoccales and formed a well supported sister clade to the uncultured archeon from the pony faeces (AB739387) and cultured strain of the *Methanomassilicoccus luminyensis*. The third cluster was the smallest one having only two sequences of the ribotype 1A distantly related, but not supported by high bootstrap value, to the strains of the *Methanomicrococcus blatticola* and *Methanosarcina barkeri*. That is why this group was attributed to Methanosarcinales. The fourth cluster grouped with the Methanomicrobiales and contained uncultured sequences of the ribotype 3A, which formed a well supported sister subcluster of the cultured strains of the species *Methanocorpusculum*.

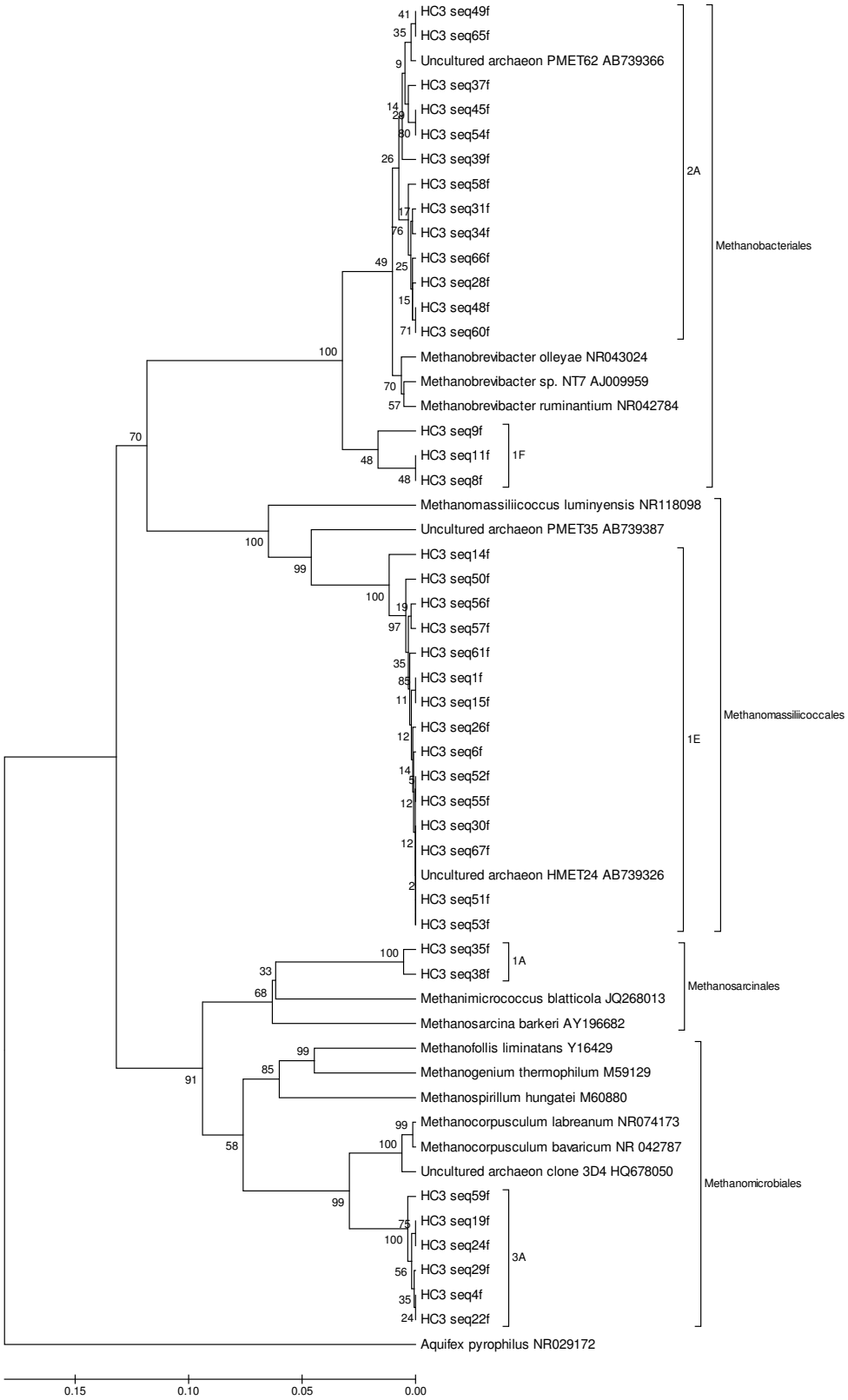


**Figure 4.3-4**. The phylogenetic tree description is reported in the following page.

**Figure 4.3-4. Phylogenetic tree of the methanogenic sequences found in the left dorsal colon of the horse and analyzed in this study. The reference sequences of methanogens were retrieved from the GenBank and phylogenetically compared with the 39 sequences generated in this study. The Aquifex pyrophilus sequence was used as an outgroup to root the tree. The evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973). The optimal tree with the sum of branch length = 1.30148772 is shown. The percentage of the replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with the branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000) and are in the units of the number of base differences per site. The analysis involved 55 nucleotide sequences. All the ambiguous positions were removed for each sequence pair. There were a total of 1608 positions in the final dataset. The evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011).**

### 4.3.5 Diversity of the archaeal 16S rDNA sequences generated from the horse right dorsal colon

A total of 68 positive clones were generated from the archaeal 16S rDNA gene cloning libraries of the horse right dorsal colon (HC4). These clones were differentiated based on the RFLP patterns and distributed into 9 ribotypes as reported in the following scheme.

| Number of clones | incidence | Ribotype of belonging |
|---|---|---|
| **29** | 43% | 2A |
| **16** | 23.5% | 3B |
| **9** | 13% | 1E |
| **7** | 10% | 1A |
| **2** | 3% | 2G |
| **2** | 3% | 3C |
| **1** | 1.5% | 1F |
| **1** | 1.5% | 2I |
| **1** | 1.5% | 3F |

40 positive clones were sequenced with the forward primer, the highest similarity of these sequences with the GenBank database entries was assessed and the phylogenetic analysis was performed. The sequences of the ribotype 2A were highly similar to the uncultured archeons obtained from the pony faeces (100% sequence similarity with the entry no. AB739366, 98% sequence similarity with the entry no. AB739369) and the horse faeces (100% sequence similarity with the entry no. AB739324). No similarity with the cultured methanogens was found. The sequences of the ribotype 2G were highly similar to the uncultured archeons obtained from the reindeer rumen (100% sequence similarity with the entry no. EU413658). The one sequence of the ribotype 1F exhibited the 100% sequence similarity with the uncultured archeon obtained from the sika deer (entry no.KC454162). The sequences of the ribotype 1E were highly similar to the uncultured archeon obtained from the horse faeces (100% sequence similarity with the entry no. AB739326). The sequences of the ribotype 1A were highly similar

to the uncultured archeon obtained from the horse faeces (100% sequence similarity with the entry no. AB739341) and the cultured methanogen *Methanomicrococcus blatticola* (98-99% sequence similarity with the entry no. JQ268013). The one sequence of the ribotype 2I exhibited the 100% sequence similarity with the uncultured archeon obtained from the horse faeces (entry no.AB739342) and the cultured methanogen *Methanomicrococcus blatticola* (99% sequence similarity with the entry no. JQ268013). The one sequence of ribotype 2F exhibited the 100% sequence similarity with the uncultured archeon obtained from the horse faeces (AB739341) and 99% similarity with the cultured *Methanomicrococcus blatticola* (JQ268013). The sequences of the ribotypes 3B and 3C were highly similar to the uncultured archeons obtained from the pony faeces (100% sequence similarity with the entry no. AB739381).

The phylogenetic analysis of all the 40 archaeal 16S rRNA gene sequences revealed that the methanogens of the horse right dorsal colon were divided into four main clusters (Figure 4.3-5). The first cluster, grouping with the Methanobacteriales, was devided into three subclusters well corresponding to the ribotypes 2A (this cluster contains the *Methanobrevibacter* strains), 2G and one sequence of 1F. The second cluster containing the sequences of the ribotype 1E grouped with the Methanomassiliicoccales and formed well supported sister clade to the first cluster. The third cluster, grouping with the Methanosarcinales, covered a subclade of sequences of the ribotype 1A and common subclade of sequences of the ribotypes 2I and 3F. The *Methanomicrococcus blaticola* was embedded between these two subclades. The forth cluster, grouping with the Methanomicrobiales and regarding the sequences generated in this study, was devided into two sister subclusters corresponding well with the sequences of the ribotypes 3C and 3B, which formed a sister cluster of the cultured strains of *Methanocorpusculum.*

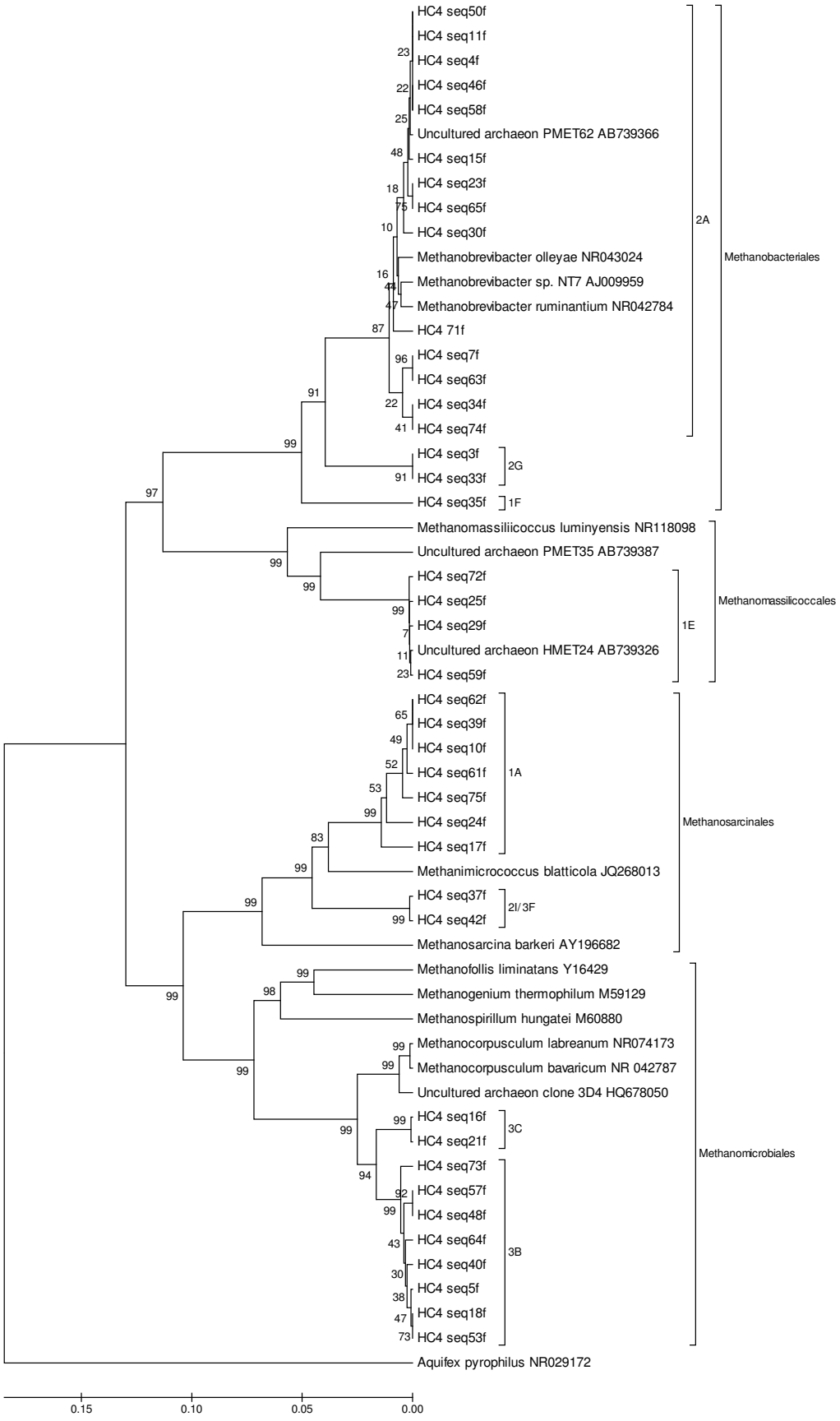


**Figure 4.3-5.** The phylogenetic tree description is reported in the following page.

**Figure 4.3-5. Phylogenetic tree of the methanogenic sequences found in the right dorsal colon of the horse and analyzed in this study. The reference sequences of methanogens were retrieved from the GenBank and phylogenetically compared with the 40 sequences generated in this study. The Aquifex pyrophilus sequence was used as an outgroup to root the tree. The evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973). The optimal tree with the sum of branch length = 1.38789357 is shown. The percentage of the replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with the branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000) and are in the units of the number of base differences per site. The analysis involved 56 nucleotide sequences. All the ambiguous positions were removed for each sequence pair. There were a total of 1622 positions in the final dataset. The evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011).**

### 4.3.6 Diversity of the archaeal 16S rDNA sequences generated from the horse faeces

A total of 61 positive clones were generated from the archaeal 16S rDNA gene cloning library of the horse faeces (HFe). These clones were differentiated based on the RFLP patterns and distributed into 8 ribotypes as reported in the following scheme.

| Number of clones | incidence | Ribotype of belonging |
|---|---|---|
| **32** | 52% | 2A |
| **9** | 15% | 3A |
| **5** | 8% | 1A |
| **7** | 11% | 4A |
| **3** | 5% | 2C |
| **2** | 3% | 1B |
| **2** | 3% | 2D |
| **1** | 2% | 2B |

41 positive clones were sequenced with the forward primer, the highest similarity of these sequences with the GenBank database entries was assessed and the phylogenetic analysis was performed. The sequences of the ribotype 2A were highly similar to the uncultured archaeon obtained from the pony faeces (99-100% sequence similarity with the entry no. AB739366). The sequences of the ribotype 3A were highly similar to the uncultured archaeons obtained from the anaerobic bioreactor (99% sequence similarity with the entry no. HQ678050), the horse faeces (100% sequence similarity with the entry no. AB739344) and the tadpole gut (100% sequence similarity with the entry no. KC841482) and from the cultured methanogens *Methanocorpusculumsp.* (98-99% sequence similarity with the entry no. AY260434) and the *Methanocorpusculum bavaricum* (99% sequence similarity with the entry no. NR042787). The sequences of the ribotype 1A showed a high similarity with the uncultured archeons from the horse faeces (100% sequence similarity with the entry no. AB739341) and with the cultured archeon *Methanomicrococcus blatticola* (98-99% sequence similarity with the entry no. JQ268013). The sequences of the ribotype 4A were highly similarity to the uncultured archeon obtained from the yak rumen (100% sequence similarity with the entries nos. JF807268, JF807269 and JF807279). The one sequence of the ribotype 2D was highly similar to the

uncultured archeons obtained from the horse faeces (100% sequence similarity with the entry no. AB739350), while the one sequence of the ribotype 2B was highly similar to the uncultured archeons obtained from the cow rumen (100% sequence similarity with the entry no. JQ179646). The sequences of the ribotype 2C were highly similar to the uncultured archeons obtained from the cow rumen (100% sequence similarity with the entry no. JF682979) and the cultured archeon *Methanobrevibacter sp.* (100% sequence similarity with the entry no. KF697721). The sequences of the ribotype 1B were highly similar to the uncultured archeons obtained from the mule faeces (100% sequence similarity with the entry no. EU863828).

The phylogenetic analysis of the 41 archaeal 16S rRNA gene sequences revealed that the methanogens of the horse faeces were divided into four main clusters (Fig. 4.3-6). The first largest cluster grouped with the Methanobacteriales and was devided into two bigger subclusters. One subcluster contained nearly identical sequences of the ribotype 2A and sequences of *Methanobrevibacter sp*. One sequence of the ribotype 1B (HFe seq26f) and one sequence of the ribotype 2C (HFe seq65f) clustered separately. The second small subcluster contained mixed sequences of the ribotypes 1B/2C/2D. The second cluster contained sequences of the ribotype 4A and grouped with the Methanomassiliicoccales. The *Methanomassiliicoccus luminyensis* represented the sister cluster of the sequences generated in this study. This grouping was supported by high bootstrap values. The third main cluster containing sequences of the ribotype 1A formed the well supported sister cluster of *Methanomicrococcus blaticola* and grouped with the Methanosarcinales. The fourth cluster containing the sequences of the ribotype 3A grouped with Methanomicrobiales and was devided into two sister subclusters. One sequence (HFe seq44f) clustered with the cultured strains of *Methanocorpusculum* and together made a sequences sister cluster of the uncultured methanogens generated in this study.

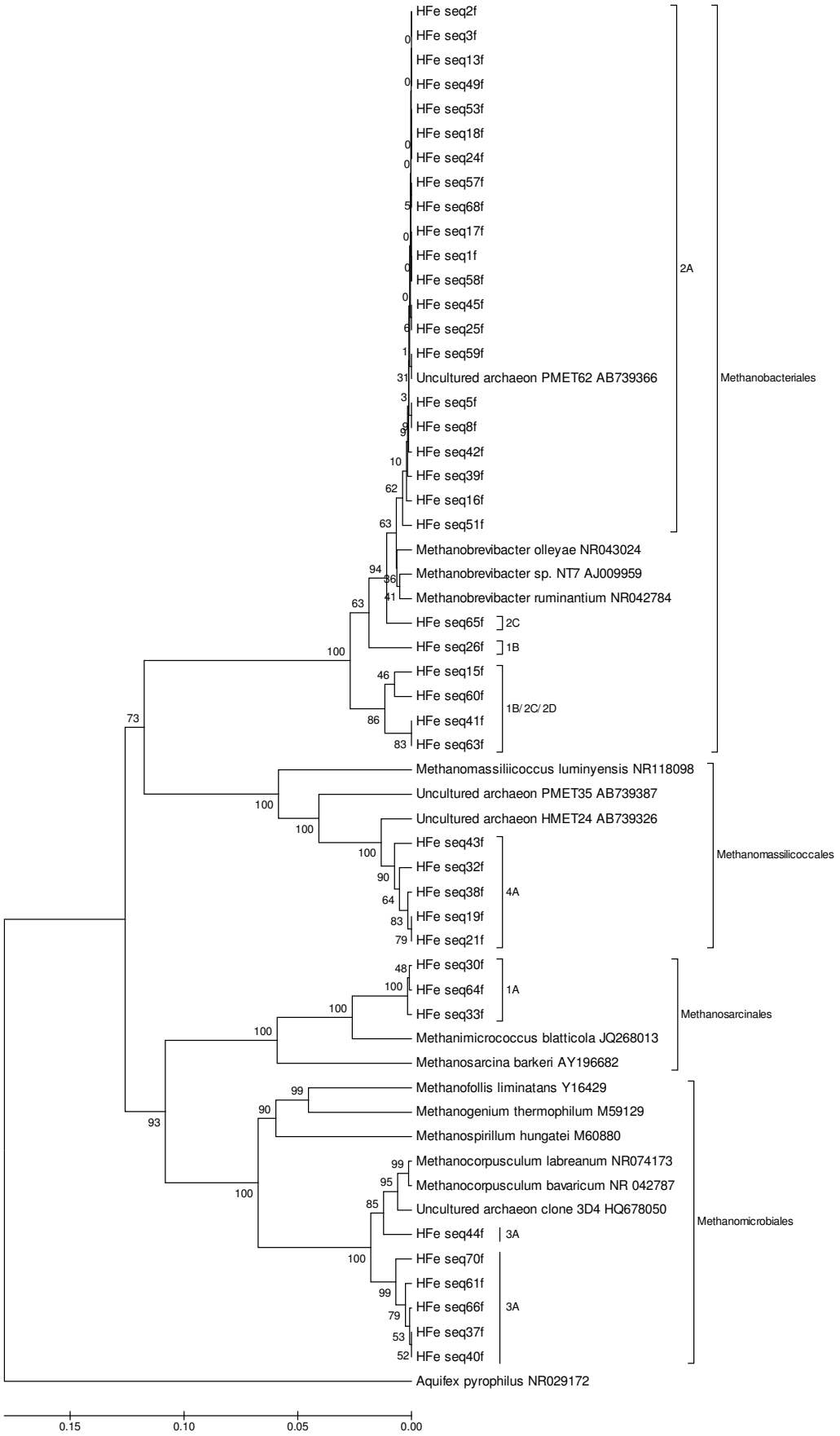


**Figure 4.3-6.** The phylogenetic tree description is reported in the following page.

**Figure 4.3-6. Phylogenetic tree of the methanogenic sequences found in the faeces of the horse and analyzed in this study. The reference sequences of methanogens were retrieved from the GenBank and phylogenetically compared with 41 sequences generated in this study. The Aquifex pyrophilus sequence was used as an outgroup to root the tree. The evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973). The optimal tree with the sum of branch length = 1.24922263 is shown. The percentage of the replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with the branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000) and are in the units of the number of base differences per site. The analysis involved 57 nucleotide sequences. All the ambiguous positions were removed for each sequence pair. There were a total of 1613 positions in the final dataset. The evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011).**

## 4.4 Phylogenetic analysis of the archaeal 16S rRNA sequences of the donkey hindgut

### 4.4.1 Diversity of the archaeal 16S rDNA sequences generated from the donkey caecum

A total of 31 positive clones were generated from the archaeal 16S rDNA gene cloning library of the donkey caecum (DCe). These clones were differentiated based on the RFLP patterns and distributed into 4 ribotypes as reported in the following scheme

| Number of clones | incidence | Ribotype of belonging |
|---|---|---|
| **16** | 52% | 3A |
| **9** | 29% | 3B |
| **4** | 13% | 1B |
| **2** | 6% | 2A |

25 positive clones were sequenced with the forward primer, the highest similarity of these sequences with the GenBank database entries was assessed and the phylogenetic analysis was performed. The sequences of the ribotype 3A were highly similar to the uncultured archeons obtained from the anaerobic bioreactor (99% sequence similarity with the entry no. HQ678050), the pony faeces (100% sequence similarity with the entry no. AB739401) and the pig caecum (100% sequence similarity with the entry no. GU391231) and to the cultured methanogens *Methanocorpusculum labreanum* (99% sequence similarity with the entry no. NR074173) and *Methanocorpusculum* sp. (99% sequence similarity with the entry no. AY260434). The sequences of the ribotype 3B were highly similar to the uncultured archeons obtained from the anaerobic bioreactor (99% sequence similarity with the entry no. HQ678050) and the pony faeces (100% sequence similarity with the entry no. AB739395) and to cultured methanogen *Methanocorpusculum* sp. (99% sequence similarity with the entry no. AY260434). The sequences of the ribotype 2A were highly similar to the uncultured archeons obtained from the rumen of buffalo (100% sequence similarity with the entry no. JF951780) and to the cultured methanogen *Methanobrevibacter ruminantium* (100% sequence similarity with the entries nos. KP123400 and KP123415). The sequences of the ribotype 1B were highly similar to the uncultured archeons obtained from the rumen of the takin (99-100% sequence similarity with the entry no. KM650110).

The phylogenetic analysis of the 25 archaeal 16S rRNA gene sequences revealed that the methanogens of the donkey caecum were divided into three main clusters (Figure 4.4-1). The first cluster grouping with the Methanomicrobiales was devided into three well supported subclusters. The upper subcluster contained sequences of the ribotype 3A, the lower subcluster contained the sequences of the ribotype 3B and the subcluster of the *Methanocorpusculum* species was embedded between them. The whole cluster and the subclusters were well supported. The second well supported cluster containing the sequences of the ribotype 1B formed the sister cluster of the cultured strain of the *Methanomassiliicoccus luminyensis*, therefore this cluster was related to the Methanomassiliicoccales. The third well supported cluster grouping with the Methanobacteriales contained the sequences of the ribotype 2A and *Methanobrevibacter ruminantium*, which formed a sister cluster of the cultured strains of *Methanobrevibacter smithii.*

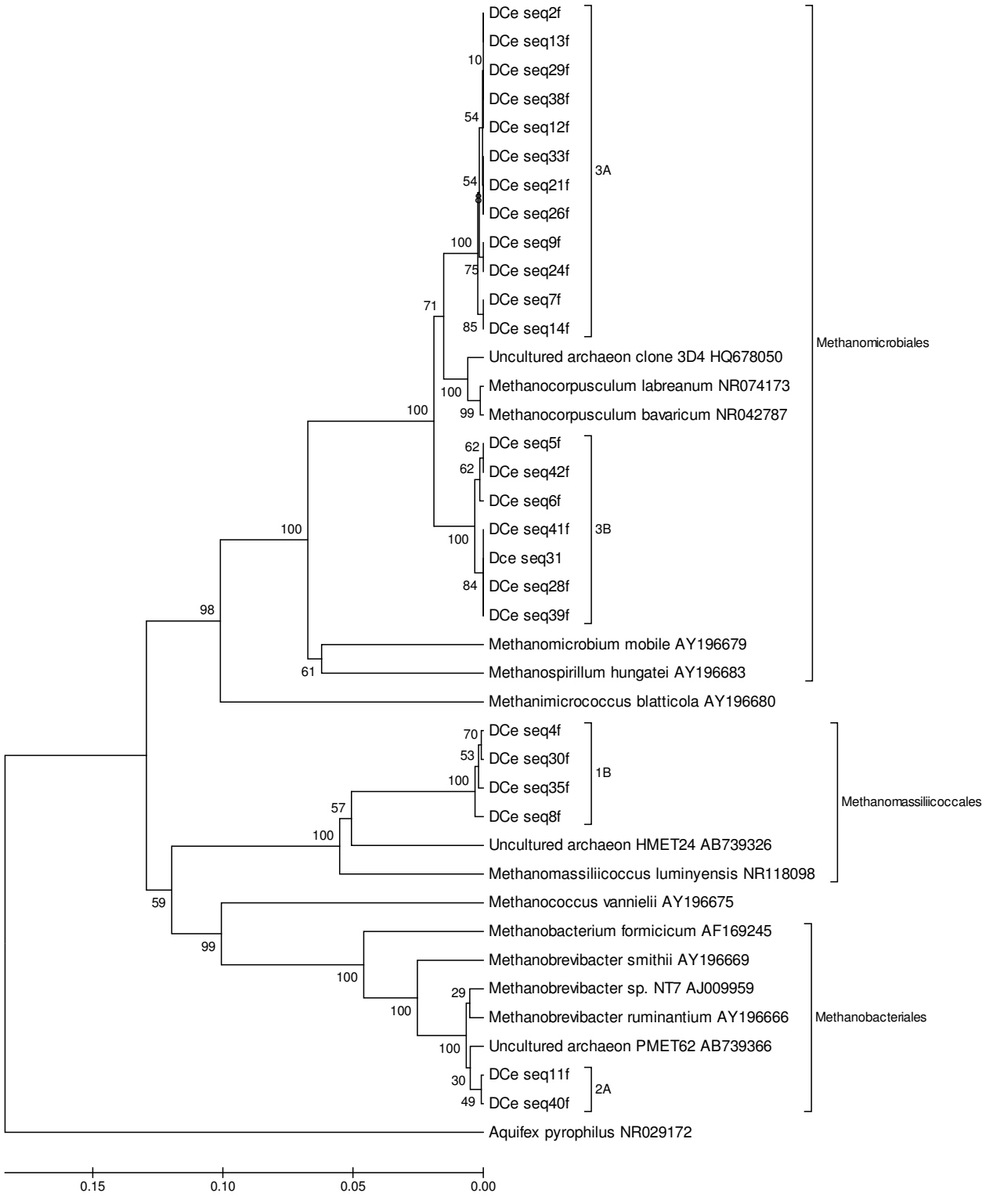


**Figure 4.4-1**. The phylogenetic tree description is reported in the following page.

**Figure 4.4-1. Phylogenetic tree of the methanogenic sequences found in the caecum of the donkey and analyzed in this study. The reference sequences of methanogens were retrieved from the GenBank and phylogenetically compared with the 25 sequences generated in this study. The Aquifex pyrophilus sequence was used as an outgroup to root the tree. The evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973). The optimal tree with the sum of branch length = 1.19721497 is shown. The percentage of the replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with the branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000) and are in the units of the number of base differences per site. The analysis involved 40 nucleotide sequences. All the ambiguous positions were removed for each sequence pair. There were a total of 1604 positions in the final dataset. The evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011).**

### 4.4.2 Diversity of the archaeal 16S rDNA sequences generated from the donkey right ventral colon

A total of 42 positive clones were generated from the archaeal 16S rDNA gene cloning library of the donkey right ventral colon (DC1). These clones were differentiated based on the RFLP patterns and distributed into 3 ribotypes as reported in the following scheme.

| Number of clones | incidence | Ribotype of belonging |
|---|---|---|
| **40** | 95% | 2A |
| **1** | 2.5% | 1F |
| **1** | 2.5% | 2D |

24 positive clones were sequenced with the forward primer, the highest similarity of these sequences with the GenBank database entries was assessed and the phylogenetic analysis was performed. The sequences of the ribotype 2A were highly similar to the uncultured archeons obtained from the pony faeces (100% sequence similarity with the entry no. AB739366) and from the rumen of buffalo (100% sequence similarity with the entry no. JF951780) and to the cultured methanogens *Methanobrevibacter ruminantium* (100% sequence similarity with the entry no. KP123415) and *Methanobrevibacter* sp. (100% sequence similarity with the entry no. AJ009959). The one sequence of the ribotype 1F was highly similar to the uncultured archeon obtained from the reindeer rumen (100% sequence similarity with the entry no. EU413598). The one sequence of the ribotype 2D exhibited the 100% sequence similarity with the uncultured archeons obtained from the reindeer rumen (entry no. EU413638).

The phylogenetic analysis of the 24 archaeal 16S rRNA gene sequences revealed that all the methanogens of the donkey right ventral colon cluster with Methanomicrobiales. (Figure 4.4-2). The well suported subcluster of the sequences of the ribotype 2A contained species of *Methanobrevibacter ruminantium*. The one sequence of the ribotytype 1F clustered separately and was embedded between the species of *Methanobrevibacter smithii* and *Methanobrevibacter formicicum.* The one sequence of the ribotype 2D clustered separately and was more closely related to the *Methanobrevibacter formicicum.*

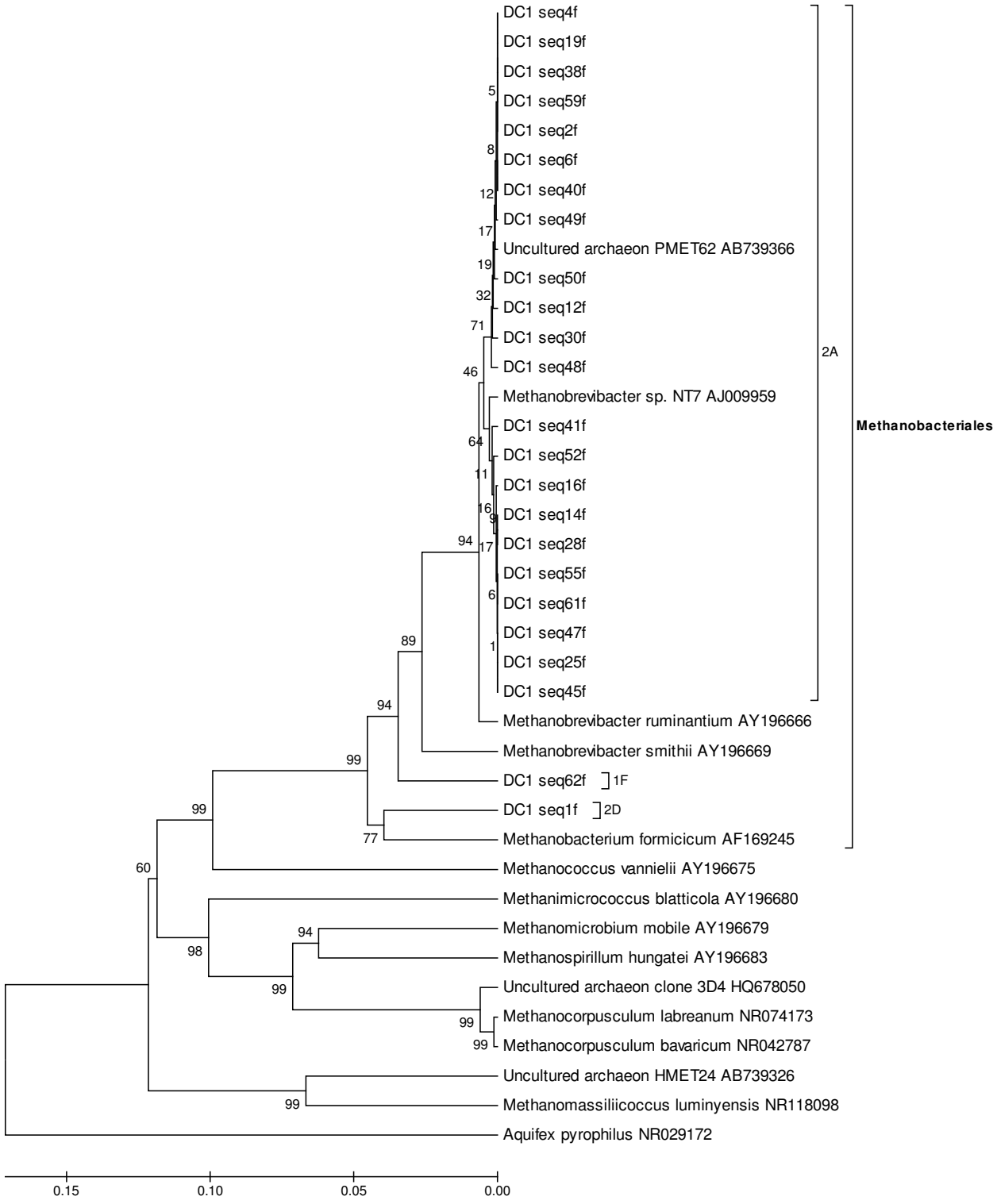


**Figure 4.4-2**. The phylogenetic tree description is reported in the following page.

**Figure 4.4-2. Phylogenetic tree of the methanogenic sequences found in the right ventral colon (RVC) of the donkey and analyzed in this study. The reference sequences of methanogens were retrieved from the GenBank and phylogenetically compared with the 24 sequences generated in this study. The Aquifex pyrophilus sequence was used as an outgroup to root the tree. The evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973). The optimal tree with the sum of branch length = 1.15937381 is shown. The percentage of the replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with the branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000) and are in the units of the number of base differences per site. The analysis involved 39 nucleotide sequences. All the ambiguous positions were removed for each sequence pair. There were a total of 1604 positions in the final dataset. The evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011).**

### 4.4.3 Diversity of the archaeal 16S rDNA sequences generated from the donkey left ventral colon

A total of 55 positive clones were generated from the archaeal 16S rDNA gene cloning library of the donkey left ventral colon (DC2). These clones were differentiated based on the RFLP patterns and distributed into 4 ribotypes as reported in the following scheme.

| Number of clones | incidence | Ribotype of belonging |
|---|---|---|
| **49** | 89% | 2A |
| **3** | 5% | 1F |
| **2** | 4% | 2N |
| **1** | 2% | 1H |

27 positive clones were sequenced with the forward primer, the highest similarity of these sequences with the GenBank database entries was assessed and the phylogenetic analysis was performed. The sequences of the ribotype 2A were highly similar to the uncultured archeons obtained from the pony faeces (100% sequence similarity with the entry no. AB739401) and the cultured methanogen *Methanobrevibacter ruminantium* (100% sequence similarity with the entry no. KP123415). The sequences of the ribotype 1F were highly similar to the uncultured archeons obtained from the buffalo rumen (99-100% sequence similarity with the entry no. JF951780). The sequences of the ribotype 2N showed high similarity with the uncultured archeons obtained from the sika deer rumen (100% sequence similar with the entry no. KC454261). The one sequence of the ribotype 1H exhibited high similarity with the uncultured archeons obtained from the cow rumen (100% sequence similarity with the entry no. JQ179712) and with the cultured methanogen *Methanomicrobium mobile* (100% sequence similarity with the entry no. AY196679).

The phylogenetic analysis of 27 archaeal 16S rRNA gene sequences revealed that the majority of the methanogens of the donkey left ventral colon clustered with Methanobacteriales, only one sequence grouped with Methanomicrobiales (Figure 4.4-3). Inside the Methanobacteriales cluster, the sequences generated in this study formed three well supported subclusters corresponding to the ribotypes 2A, 2N and 1F. The sequences of the ribotype 2A formed a sister cluster of *Methanobrevibacter* species, including *Methanobrevibacter ruminantium*. The sequences of the ribotypes 2N and 1F clustered separately and the strain *Methanobacterium*

*formicicum* was embedded between these two subclusters. The single sequence of ribotype 1H (DC2 seq104f) clustered separately together with the *Methanomicrobium mobile* (Methanomicrobiales).

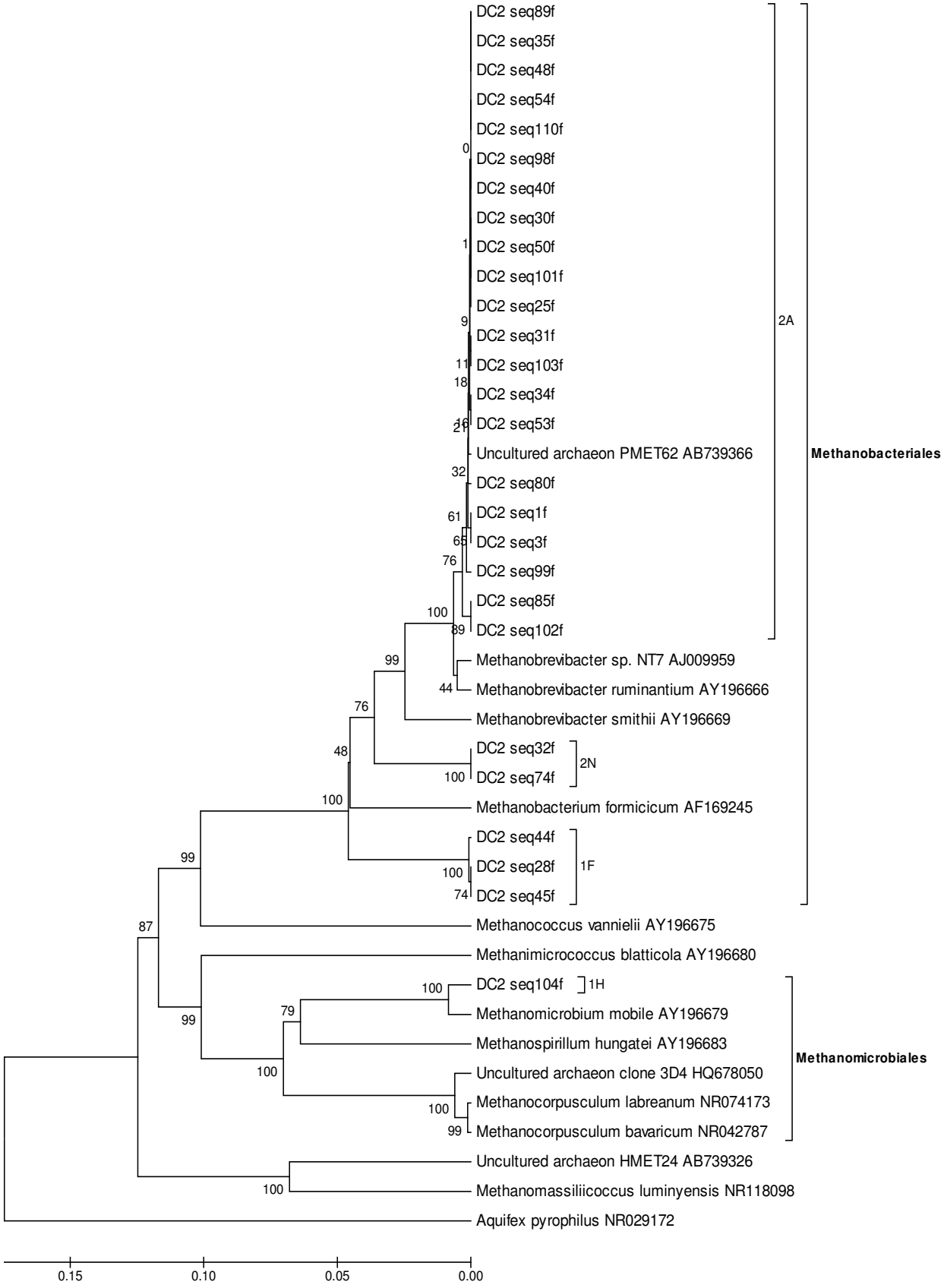


**Figure 4.4-3**. The phylogenetic tree description is reported in the following page.

**Figure 4.4-3. Phylogenetic tree of the methanogenic sequences found in the left ventral colon (LVC) of the donkey and analyzed in this study. The reference sequences of methanogens were retrieved from the GenBank and phylogenetically compared with the 27 sequences generated in this study. The Aquifex pyrophilus sequence was used as an outgroup to root the tree. The evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973). The optimal tree with the sum of branch length = 1.18317973 is shown. The percentage of the replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with the branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000) and are in the units of the number of base differences per site. The analysis involved 42 nucleotide sequences. All the ambiguous positions were removed for each sequence pair. There were a total of 1604 positions in the final dataset. The evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011).**

### 4.4.4 Diversity of the archaeal 16S rDNA sequences generated from the donkey left dorsal colon

A total of 55 positive clones were generated from the archaeal 16S rDNA gene cloning library of the donkey left dorsal colon (DC3). These clones were differentiated based on the RFLP patterns and distributed into 3 ribotypes as reported in the following scheme.

| Number of clones | incidence | Ribotype of belonging |
|---|---|---|
| **38** | 69% | 3A |
| **12** | 22% | 3B |
| **5** | 9% | 1B |

31 positive clones were sequenced with the forward primer, the highest similarity of these sequences with the GenBank database entries was assessed and the phylogenetic analysis was performed. The sequences of the ribotype 3A were highly similar to the uncultured archeons obtained from the pig caecum (100% sequence similarity with the entry no. GU391231) and the anaerobic bioreactor (99% sequence similarity with the entry no. HQ678050) and to the cultured methanogens *Methanocorpusculum labreanum* (99% sequence similarity with the entry no. NR029086) and *Methanocorpusculum* sp. (99% sequence similarity with the entry no. AY260434). The sequences of the ribotype 3B exhibited the same affiliation as 3A, because they were also highly similar to the uncultured archeons obtained from the pig caecum (100% sequence similarity with the entry no. GU391231) and the anaerobic bioreactor (99% sequence similarity with the entry no. HQ678050) and to the cultured methanogen *Methanocorpusculum* sp. (99% sequence similarity with the entry no. AY260434). The sequences of ribotype 1B exhibited high similarity with the uncultured archeons obtained from the pig colon (100% sequence similarity with the entry no. JX522655), the cattle rumen (100% sequence similarity with the entry no. HQ616013) and only 96% sequence similarity with the archeon from the rumen of the takin (entry no. KM650110).

The phylogenetic analysis of the 31 archaeal 16S rRNA gene sequences revealed that the methanogens of the donkey left dorsal colon were divided into two main clusters (Figure 4.4-4). The first large cluster grouped with the Methanomicrobiales and was devided into two subclusters. Uppersubcluster contained the sequences of the ribotype 3A, which were however mixed with some sequences of the ribotype 3B. Five sequences of the ribotype 3B clustered

separately in a well supported clade. Between these two subclustres the cluster of the species of *Methanocorpusculum* was embedded. The second cluster grouped with the Methanomassiliicoccales, corresponded well with the sequences of the ribotype 1B and formed a sister cluster to the cultured strain of *Methanomassiliicoccus luminyensis*.

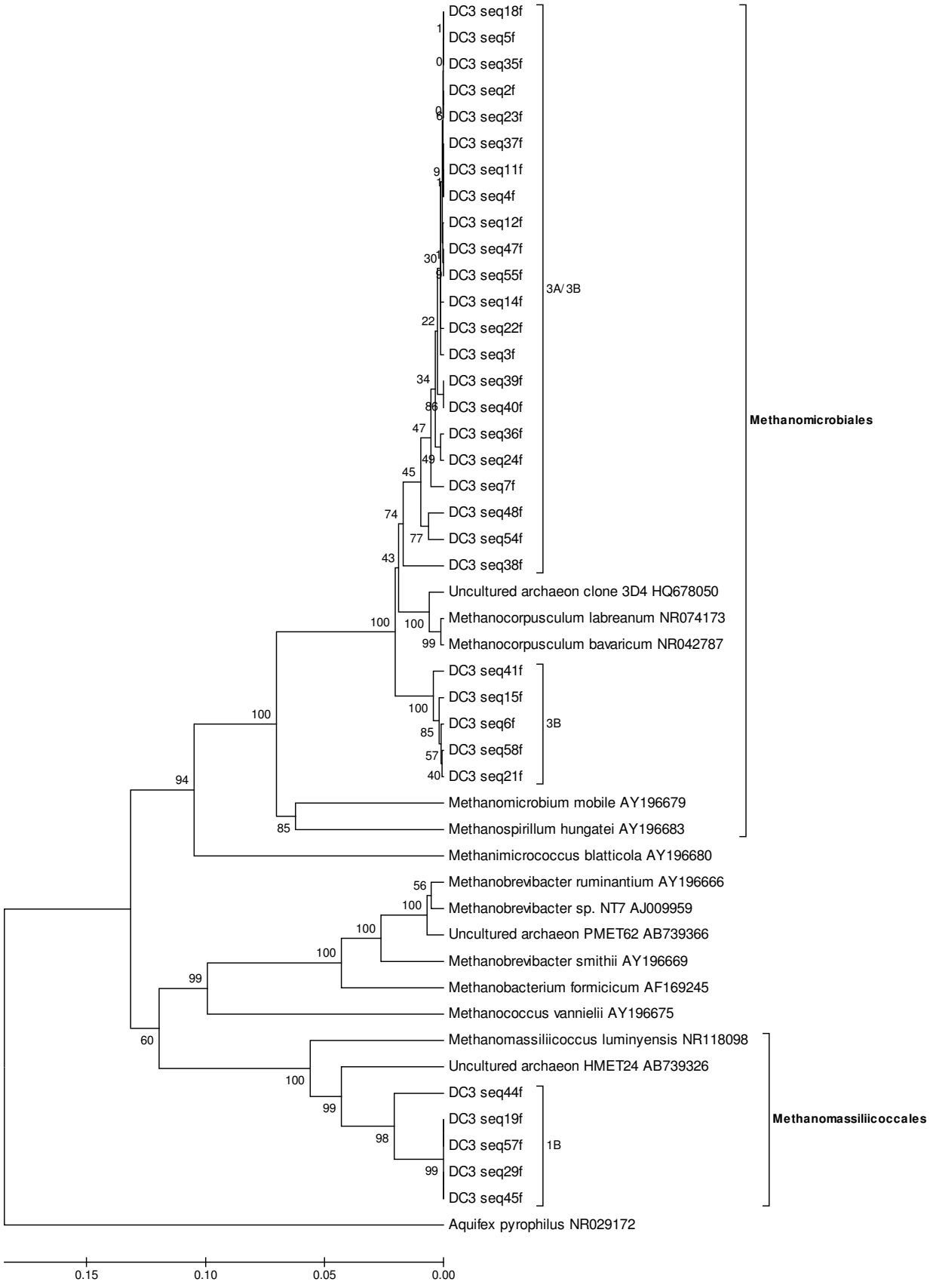


**Figure 4.4-4.** The phylogenetic tree description is reported in the following page.

**Figure 4.4-4. Phylogenetic tree of the methanogenic sequences found in the left dorsal colon (LDC) of the donkey and analyzed in this study. The reference sequences of methanogens were retrieved from the GenBank and phylogenetically compared with the 31 sequences generated in this study. The Aquifex pyrophilus sequence was used as an outgroup to root the tree. The evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973). The optimal tree with the sum of branch length = 1.24546777 is shown. The percentage of the replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with the branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000) and are in the units of the number of base differences per site. The analysis involved 46 nucleotide sequences. All the ambiguous positions were removed for each sequence pair. There were a total of 1604 positions in the final dataset. The evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011).**

### 4.4.5 Diversity of the archaeal 16S rDNA sequences generated from the donkey right dorsal colon

A total of 59 positive clones were generated from the archaeal 16S rDNA gene cloning library of the donkey right dorsal colon (DC4). These clones were differentiated based on the RFLP patterns and distributed into 3 ribotypes as reported in the following scheme.

| Number of clones | incidence | Ribotype of belonging |
|---|---|---|
| **48** | 81% | 3A |
| **10** | 17% | 3B |
| **1** | 2% | 1H |

26 positive clones were sequenced with the forward primer, the highest similarity of these sequences with the GenBank database entries was assessed and the phylogenetic analysis was performed. The sequences of the ribotype 3A were highly similar to the uncultured archeons obtained from the pig caecum (100% sequence similarity with the entry no. GU391231) and the anaerobic bioreactor (99% sequence similarity with the entry no. HQ678050) and to the cultured methanogens *Methanocorpusculum labreanum* (99% sequence similarity with the entry no. NR029086) and *Methanocorpusculum sp.* (99% sequence similarity with the entry no. AY260434). The sequences of the ribotype 3B were highly similar to the uncultured archeons obtained from the pony faeces (100% sequence similarity with the entry no. AB739401) and the anaerobic bioreactor (99% sequence similarity with the entry no. HQ678050) and to the cultured methanogen *Methanocorpusculum sp*. (99% sequence similarity with the entry no. AY260434). The one sequence of the ribotype 1H exhibited high similarity with the uncultured archeon obtained from the cow rumen (99% sequence similarity with the entry no. AY464786) and with the cultured methanogen *Methanomicrobium mobile* (99% sequence similarity with the entry no. AY196679).

The phylogenetic analysis of the 26 archaeal 16S rRNA gene sequences revealed that all the methanogens of the donkey right dorsal colon grouped with Methanomicrobiales. The main cluster was divided into two subclusters (Figure 4.4-5). The upper large well supported subcluster, containing mixture of the sequences of the ribotypes 3A/3B, was split into three subclades showing a close relation to the *Methanocorpusculum* species, but only middle subclade of the cultured *Methanocorpusculum* strains was well supported. The lower small well

supported subcluster contained only one sequence of the ribotype 1H (DC4 seq45f) related to the *Methanomicrobium mobile.*

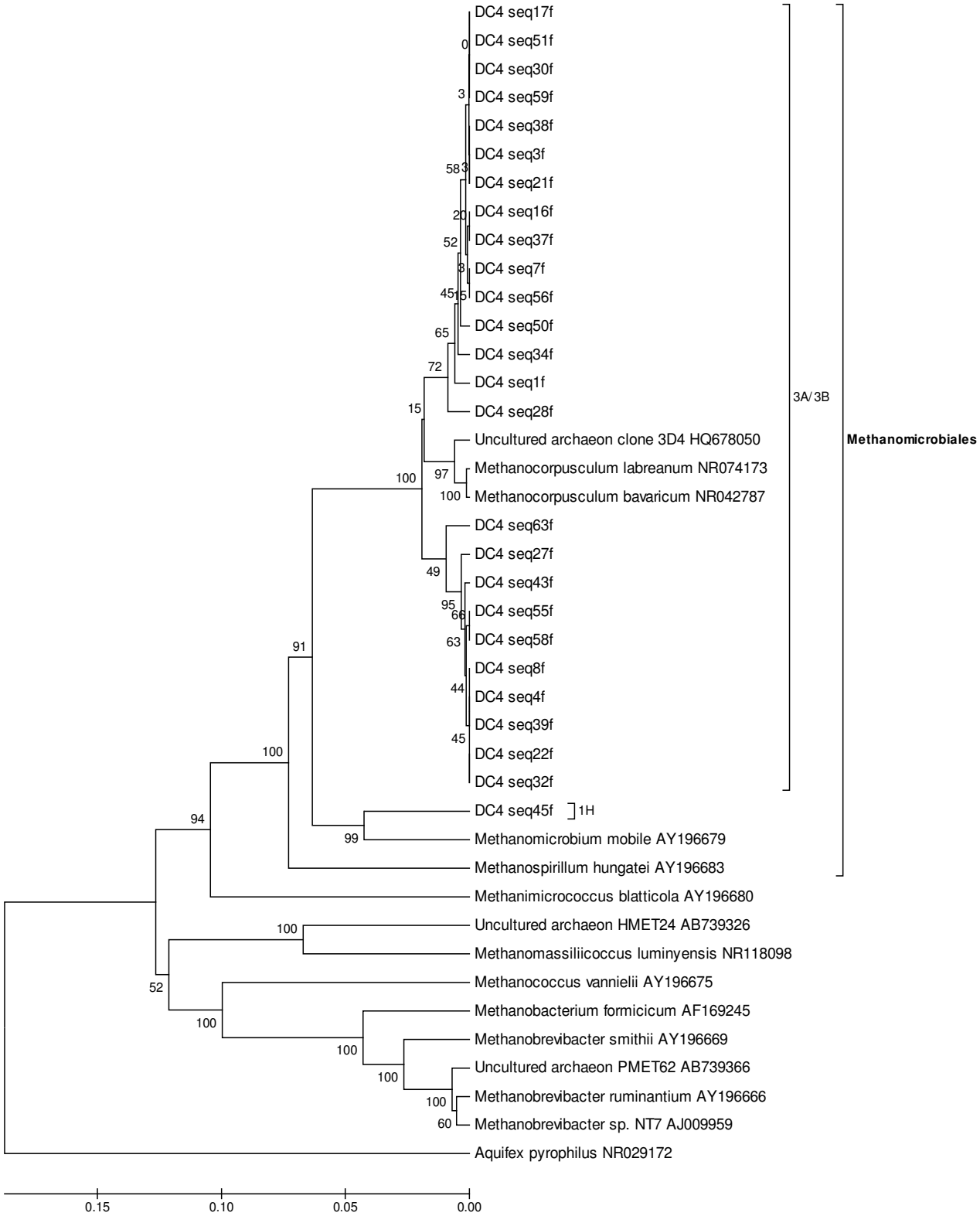


**Figure 4.4-5**. The phylogenetic tree description is reported in the following page

**Figure 4.4-5. Phylogenetic tree of the methanogenic sequences found in the right dorsal colon (RDC) of the donkey and analyzed in this study. The reference sequences of methanogens were retrieved from the GenBank and phylogenetically compared with the 26 sequences generated in this study. The Aquifex pyrophilus sequence was used as an outgroup to root the tree. The evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973). The optimal tree with the sum of branch length = 1.23915556 is shown. The percentage of the replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with the branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000) and are in the units of the number of base differences per site. The analysis involved 41 nucleotide sequences. All the ambiguous positions were removed for each sequence pair. There were a total of 1605 positions in the final dataset. The evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011).**

### 4.4.6 Diversity of the archaeal 16S rDNA sequences generated from donkey faeces

A total of 97 positive clones were generated from the archaeal 16S rDNA gene cloning library of the donkey faeces (DFe). These clones were differentiated based on the RFLP patterns and distributed into 5 ribotypes as reported in the following scheme.

| Number of clones | incidence | Ribotype of belonging |
|---|---|---|
| **59** | 61% | 3A |
| **27** | 28% | 3B |
| **8** | 8% | 1B |
| **2** | 2% | 1C |
| **1** | 1% | 2A |

37 positive clones were sequenced with the forward primer, the highest similarity of these sequences with the GenBank database entries was assessed and the phylogenetic analysis was performed. The sequences of the ribotype 3A were highly similar to the uncultured archeons obtained from the anaerobic bioreactor (99% sequence similarity with the entry no. HQ678050) and the pig caecum (100% sequence similarity with the entry no. GU391231) and to the cultured methanogens *Methanocorpusculum labreanum* (100% sequence similarity with the entry no. NR074173), *Methanocorpusculum* sp. (100% sequence similarity with the entry no. AY260434) and *Methanocorpusculum aggregans* (99% sequence similarity with the entry no. NR117749). The sequences of the ribotype 3B were highly similar to the uncultured archeons obtained from the anaerobic bioreactor (99% sequence similarity with the entry no. HQ678050) and the pony faeces (100% sequence similarity with the entries nos. AB739336, -381, -384, 394, -395, -401), and to the cultured methanogen *Methanocorpusculum* sp. (99% sequence similar with the entry no.AY260434). The sequences of the ribotype 1B were highly similar to the uncultured archeons obtained from the rumen of the takin (100% sequence similarity with the entry no. KM650110) and the pig colon (100% sequence similar with the entry no. JX522655). The sequences of the ribotype 1C (DFe seq89f, DFe seq140f) showed similarity with the uncultured archeons from the pony faeces (96-99% sequence similar with the entry no. AB739382). The one sequence of ribotype 2A (DFe seq149f) was similar to uncultured archeon obtained from the orangutan colon (97% sequence similarity with the entry no. JN192495,

however low query) and to the cultured methanogen *Methanobacterium sp.* (96% sequence similarity with the entry no. DQ677518).

The phylogenetic analysis of the 37 archaeal 16S rRNA gene sequences revealed that the methanogens of the donkey faeces were divided into three main clusters (Figure 4.4-6). The first biggest cluster grouping with the Methanomicrobiales was devided into three subclusters. Two upper subclusters contained the sequences of the ribotypes 3A/3B and the subcluster of the *Methanocorpusculum* species was embedded between them. The second cluster contained only one sequence of the ribotype 2A, which was embedded between the *Methanobacterium formicicum* (Methanobacteriales) and the *Methanococcus vannielii* (Methanococcales), two species belonging to different order. The third well supported cluster, containing the sequences of the ribotype 1B, grouped with the Methanomassiliicoccales and formed a sister cluster of the strain *Methanomassiliicoccus luminyensis*.

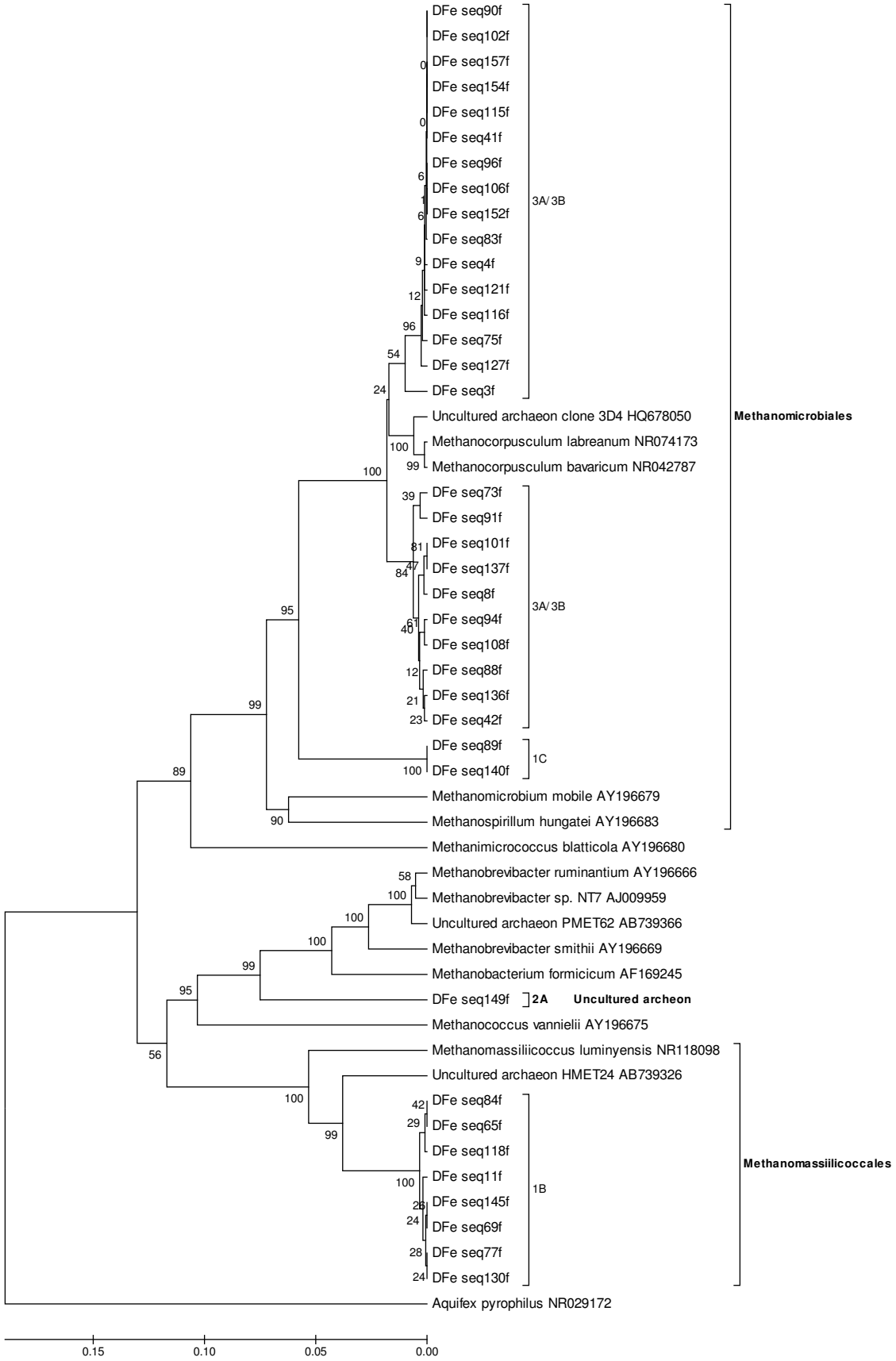


**Figure 4.4-6**. The phylogenetic tree description is reported in the following page.

**Fig. 4.4-6. Phylogenetic tree of the methanogenic sequences found in the faeces of the donkey and analyzed in this study. The reference sequences of methanogens were retrieved from the GenBank and phylogenetically compared with the 37 sequences generated in this study. The Aquifex pyrophilus sequence was used as an outgroup to root the tree. The evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973). The optimal tree with the sum of branch length = 1.36487241 is shown. The percentage of the replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with the branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000) and are in the units of the number of base differences per site. The analysis involved 52 nucleotide sequences. All the ambiguous positions were removed for each sequence pair. There were a total of 1604 positions in the final dataset. The evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011).**

# 5. DISCUSSION

## 5.1 Discussion

Methanogens are members of the microbial communities inhabiting the large intestine of equine. Non-methanogenic populations (bacteria, protozoa, fungi) ferment substrates to short chain volatile fatty acids, $H_2$ and $CO_2$. Methanogens then use the $H_2$ to reduce $CO_2$ to $CH_4$. Methanol and methylamines derived from dietary sources can be also utilized by methanogens, however these products are used for the production of $CH_4$ in lesser amounts. The microbial population plays an important role on the overall nutritional and health status of animals. For this reason, the methanogens diversity in the equine GIT is important for understanding the microbial ecosystem and for improving the digestive processes in their hindgut. To date, only limited information is available on the methanogen population of the horse's hindgut (Lwin and Matsui, 2014, Fernandes *et al.* 2014), while the methanogenic population of the donkey hindgut is unknown.

Several studies have been conducted to analyze the composition and population size of methanogens mainly in ruminant animals. These studies have reported that the most common species of methanogens isolated and identified from the rumen are the strains of *Methanobrevibacter, Methanomicrobium, Methanobacterium* and *Methanosarcina* (Jarvis *et al.*, 2000; Steinfeld *et al.*, 2006; Wolin *et al.*, 1997). Regarding the archaeal diversity of equidae, the species of *Methanobrevibacter* found as the common methanogen in horse faeces (Jensen, 1996; Lin and Miller, 1998; Yamano *et al.*, 2008; Shepherd *et al.*, 2012) has been mostly obtained as the "by-product" of the studies of the equine bacterial 16S rRNA. However, Lwin and Matsui (2014) described the *Methanobrevibacter* (order Methanobacteriales) as the less numerous species in the faeces of adult horses and the Methanomicrobiales, represented by *Methanocorpusculum labreanum*, as the predominant order. Recently, the prevalence of these two archaeal clades, Methanobrevibacter and Methanocorpusculum, in the equine faeces has been described by Fernandes *et al.* (2014). The incidence of

*Methanocorpusculum labreanum* was reported by DGGE analysis in all the segments of the horse hindgut (Fliegerova *et al.* 2016), however the prevalence of this strain was shown mainly in caecum and faeces, while the colon was dominated by uncultured methanogens. These preliminary results indicated the influence of the gut region on the abundance and composition of the equine intestinal archaeal population and induced this our more detailed study of methanogens along the equine hindgut. In the present thesis, we studied the diversity of the methanogens along the horse and donkey hindgut by the methods of molecular biology using the archaeal 16S rRNA gene as marker.

The RFLP analysis of 345 sequences generated from the horse hindgut and 335 sequences generated from the donkey hindgut revealed interesting differences among the different parts of the digestive tract of both the animals and diversity differences between these two animals. The methanogens diversity in the donkey digestive tract was much less when compared to that of the horse, however the three dominant ribotypes (2A, 3A, 3B) were present in both the animals, even if their frequency was different.

Representatives of each ribotype were selected and subjected to the sequencing. The phylogenetic analysis of these cloned archaeal 16S rRNA gene sequences proved the findings obtained by the RFLP analysis and brought more detailed information about the methanogens affiliation of the horse and donkey hindgut. The phylogenetic analysis showed that the methanogens clones of the horse hindgut were classified in 4 clades: Methanobacteriales (corresponding with the ribotype 2A), Methanomicrobiales (corresponding with the ribotypes 3A and 3B), Methanomassiliicoccales and Methanosarcinales. The donkey hindgut methanogens clones were classified in 3 clades: Methanobacteriales, Methanomassiliicoccales and Methanomicrobiales. However the distribution of the clones belonging to these clades was very different between the two species and among the different parts of the digestive tract of each animal.

Among the 257 sequences of the methanogens generated from the horse hindgut nearly half of them (46%) was highly similar (99-100%) to the cultivated methanogens, especially among these 54% of them were similar to the Methanocorpusculum and 36% to the Methanobrevibacter, less sequences were similar to the *Methanomicrococcus*

*blaticola* (10%). Here the generated sequences similar to the Methanobrevibacter have been already detected in the horse and pony faeces (Lwin and Matsui, 2014), while the sequences affiliated with Methanocorpusculum strains were mostly detected in the anaerobic bioreactor (Talbot *et al.*, unpublished), pig caecum (Dhali *et al.* unpublished) and saline lake (Jiang *et al.*, unpublished) but only rarely in the horse, pony (Lwin and Matsui, 2014) and even in the tadpole (Chang *et al.* unpublished). The distribution of the sequences along the hindgut was dependent on the part of the digestive tract. The *Methanobrevibacter* sequences dominated in all parts of the colon and in the faeces, while the *Methanocorpusculum* sequences entirely dominated in the caecum (HCe). The absence of the *Methanobrevibacter* and the dominance of the *Methanocorpusculum* species in the horse caecum is an interesting finding, which can reflect the distinct metabolic processes of the caecum. Few sequences of *Methanomicrococcus blaticola* were detected in the right dorsal colon (HC4) and faeces (HFe).

More than half of the sequences from the horse hindgut (54%) were identified as uncultured methanogens, which have been already found mostly in the horse and pony faeces (Lwin and Matsui, 2014), only 11% of these sequences were highly similar to the uncultured methanogenic clones identified in the rumen of yak (Huang *et al.* 2012), cow (King *et al.,* 2011; Kong *et al.*, 2013), buffalo (Yang *et al.* unpublished), goat (Pei *et al.*, unpublished), deer (Li *et al.*, 2013; Sundset *et al.*, 2009) and in the faeces of rhino (Luo *et al.*, 2013). 4 sequences from the horse caecum and left ventral colon exhibited a very low similarity (86-87%) with the hits from GenBank and can be considered as new unknown genus or species of methanogens.

Among the 165 sequences of the methanogens generated from the donkey hindgut the vast majority of them (87%) was highly similar (99-100%) to the cultivated methanogens, especially among these, 68% to the *Methanocorpusculum* and 31% to the *Methanobrevibacter* and only few of the sequences exhibited similarity with the *Methanomicrobium mobile* (1%).

The *Methanocorpusculum* strains or clones with high similarity to the generated sequences from donkey were mostly detected in the anaerobic bioreactor (Talbot *et al.*, unpublished) and in the pig caecum (Dhali *et al.*, unpublished), less in the pony faeces

(Lwin and Matsui, 2014), while similar *Methanobrevibacter* strains or clones were detected in the rumen of buffalo (Franzolin *et al*., 2012) as well as in the pony faeces (Lwin and Matsui, 2014). The distribution of the sequences along the hindgut was dependent on the part of the donkey digestive tract. As in the horse, also in the donkey the *Methanocorpusculum* sequences entirely dominated in the caecum, but their prevalence was moreover detected in the left (DC3) and right dorsal colon (DC4) and in the faeces (DFe). On the other hand, the *Methanobrevibacter* sequences dominated in the right (DC1) and in the left ventral colon (DC2) and have not been detected at all in the dorsal colon (DC3 and DC4) and in the faeces (DFe). The dominance of the *Methanocorpusculum* clones in the caecum, dorsal colon and faeces and the dominance of the Methanobrevibacter clones in the ventral colon also indicate the differences in the metabolic activities among the different hindgut segments of the donkey. Surprisingly, no similarity with the GenBank methanogenic sequences retrieved from the horse hindgut was found. Based on the BLAST search, the archaeal sequences generated in this study from the donkey hindgut were similar to those of the pony faeces, but not any similarity with the GenBank sequences from the horse faeces was found.

The uncultured methanogens identified in the donkey hindgut formed a minority of the sequences (13%) and exhibited a sequences similarity ranging from 96 to 100% to the uncultured archeons detected already in the rumen of the takin (Yang *et al.*, unpublished), cattle (Tan *et al.*, 2011), buffalo (Franzolin *et al.*, unpublished), deer (Sundset *et al.*, 2009) and in the pig (Luo, unpublished) and orangutan (Facey and Wright, unpublished) colon. Only two of these uncultured sequences have been described in the pony faeces (Lwin and Matsui, 2014).

The presence of the Methanomicrobiales as the major phylotype in both the equids digestive tracts is in agreement with the research of Lwin and Matsui (2014), who reported that in the hindgut of horse and pony, the order Methanomicrobiales, represented by the *Methanocorpusculum spp*., was the most abundant group. Also in the hindgut of captive white rhinoceroses Luo *et al.* (2013) showed that the predominant methanogens were related to the genus *Methanocorpusculum* and this study was the first to report the methanogens closely related to the *Methanocorpusculum labreanum* as the predominant phylotype in the gastrointestinal tract of the animals. Horses, like

rhinoceros, are typical hindgut fermenters and the distribution of this species in their hindgut may likely be due to the digestive physiology of the hindgut and may play an unusual function for the digestion of the dietary fibers (Luo *et al.*, 2013). The phylogenetic analysis from the hindgut of the white rhinoceroses indicated that the methanogen species were more similar to those of the horse's hindgut (Luo *et al.*, 2013). The Methanogens of the Methanomicrobiales were however also prevalent in the rumen of sheep (approximately 54%; Yanagita *et al.* 2000) and cattle (21–54%; Tajima *et al.,* 2001); they were also dominant in the Korean native cattle (Shin *et al.,* 2004) and Murrah buffalo (Chaudhary *et al.,* 2009). The *Methanocorpusculum strains* produce methane from $H_2$ and $CO_2$, or can use formate, but not methanol and methylamines (Paul *et al.,* 2012).

The presence of the Methanobacteriales, represented by the *Methanobrevibacter* spp., has been reported as the most predominant species of methanogens in the GI tracts of many animals (Garcia *et al*., 2000). Also in horses, Lin and Miller (1998) and Lwin and Matsui (2014) recovered sequences showing 99.3% sequence similarity to the 16S rRNA gene of the *Methanobrevibacter smithii*. Studies on ruminants and on monogastric animals have indicated that *Methanobrevibacter smithii* affects the digestion efficiency of the dietary polysaccharides (Denman *et al.,* 2007). *Methanobrevibacter smithii* is also considered the predominant methanogenic archaea in the human GI tract (Saengkerdsub and Ricke, 2013). *Methanobrevibacter* species are hydrogenotrophic and they can produce methane using hydrogen and carbon dioxide as substrates. All *Methanobrevibacter spp.,* except the type strain *Methanobrevibacter arboriphilus* originating from decaying cottonwood tissue, were isolated from GI tracts (Miller and Lin, 2002; Rea *et al*., 2007). In the bovine rumen contents *Methanobrevibacter ruminantium* has been considered the predominant methanogen (Whitford *et al.,* 2001).

The sequences of many uncultured clones generated in this study (20% of the horse clones and 12% of the donkey clones) phylogenetically formed a sister clade of the *Methanomassiliicoccus luminyensis* and therefore were affiliated to the order Methanomassilicoccales (former name Methanoplasmatales). They were found as the second dominant clade in the faeces of thoroughbred horses (Lwin and Matsui, 2014).

Methanomassiliicoccales, the seventh order of methanogens, have been identified in diverse anaerobic environments including the gastrointestinal tracts (GIT) of humans and animals and may contribute significantly to the methane emission and global warming. The Methanomassiliicoccales affiliated 16S rRNA gene sequences have been recovered from natural and artificial anoxic habitats, e.g. in the rice paddy fields (Grosskopf *et al.,* 1998), anaerobic digestors (Godon *et al.,* 1997) and in the gastro-intestinal tracts (GIT) of various ruminant and non-ruminant animals, such as cattle (Tajima *et al.,* 2001), sheep (Wright *et al*., 2004), reindeer (Sundset *et al*., 2009), yak (Huang *et al.,* 2012). The Methanomassiliicoccales are phylogenetically distant from all the other orders of methanogens and belong to a large evolutionary branch composed by lineages of non-methanogenic archaea (Borrel *et al.,* 2014). All the culture-based studies agreed on a common methanogenic pathway relying on the obligate dependence of the strains on an external $H_2$ source to reduce the methyl-compounds into methane. The restriction to this metabolism was previously only observed in two methanogens from the digestive tract (*Methanosphaera stadtmanae* and *Methanomicrococcus blatticola*) and considered an exception. The apparently large distribution of this obligate metabolism among this novel order of methanogens turns this exception into one of the important pathways among over all the methanogens. Several recent studies showed that Methanomassiliicoccales have an energy metabolism distinct from the other methanogens. One of the few general characteristics of the order Methanomassiliicoccales is that the production of methane is dependent on hydrogen, methanol and methylamines. All the strains lack the genes encoding the entire $C_1$ pathway for the reduction of $CO_2$ to methyl-CoM, but possess the complete gene sets for the utilization of methanol and methylamines (Borrel *et al*., 2013). In this study *Methanomassilicoccus-like* sequences were more common in the horse hindgut, where they were found in all the segments. A lower number of *Methanomassilicoccus-like* sequences were detected in the donkey, where they were found only in the caecum, left dorsal colon and faeces. These sequences form a sister cluster of *Methanomassiliicoccus luminyensis*, which indicate that they can represent a new type of strains inside the Methanomassiliicoccales order.

5% of the sequences found only in the horse formed phylogenetically the sister clade(s) of *Methanimicrococcus blaticola* and therefore were affiliated to the order Methanosarcinales. *Methanimicrococcus blaticola* is a metabolically non-versatile member of the Methanosarcinales and it is specialised in methane formation by the hydrogen-dependent reduction of methanol, monomethyl-, dimethyl- or trimethylamine. This methanogen was isolated from the hindgut of the cockroach (Sprenger *et al*., 2000), where it was attached to the hindgut wall. The presence of the clones belonging to the Methanosarcinales in the horse hindgut could be explained by the fact that recent studies have revealed that the composition of methanogens in animal intestinal tracts can vary with different diets.

High-fiber diets may increase either the methanogenic diversity or the level of organisms. It was reported by Wright *et al.* (2004) that pasture-grazed sheep exhibited greater methanogens diversity than sheep fed either oat hay or lucerne hay. An increase in the fiber content of the diets has shown an increasing availability of methanol and methylamine in the GI tract. Methanol is produced by the hydrolysis of methyl esters from pectins, which are abundant polysaccharides in plants. *Methanosarcinales* can utilize methanol as a methanogenic substrate. Jeyanathan *et al.* (2011) reported that *Methanosarcina spp.* was one of the dominant groups when the red deers were grazed on summer pasture. Also Pol and Demeyer (1988) demonstrated that sheep fed a hay-concentrate diet increased their rate of methanogenesis from methanol, which is a substrate for *Methanosarcina spp*.

The results presented in this study show that even if the diversity of methanogens in the hindgut of equidae is dependent on the part of gastrointestinal tract, the hydrogenotrophic pathway is the main metabolic route to produce methane in the digestive tract of horse and donkey. The biological methane formation from $H_2$ and $CO_2$ ($\Delta G^{\circ}{}' = -131$ kJ/mol) is not only a quantitatively important process but possibly one of the oldest (Balch *et al.*, 1979). The hydrogenotrophic methanogens, represented in both the studied animals by clones affiliated to the Methanomicrobiales and Methanobacteriales, are quite restricted, using $H_2$, formate, or for a few species, certain alcohols as electron donors for the $CO_2$ reduction to $CH_4$. However, the methylotrophic

methanogens have been also detected in both the equids. In the donkey the clones affiliated to Methanomasiliicocales represented 12%, but in the horse the Methanomasiliicocales together with Methanosarcinales represented as much as 25%. The methylotrophic methanogens are relatively versatile, as their substrate repertoire for methanogenesis includes $H_2$ and $CO_2$, acetate, methyl compounds, such as methanol and methylamines. The methylotrophic methanogens are distinct from hydrogenotrophic methanogens in their use of electron bifurcation as an energy-conserving step in methanogenesis from $CO_2$ (Thauer *et al.,* 2008). By coupling the final, methane-producing step to the initial, $CO_2$-reducing step, electron bifurcation renders the pathway cyclic. The cyclic model of methanogenesis was recently named the Wolfe cycle in honor of the contributions of Ralph S. Wolfe. The cyclic nature of the pathway can explain in part why hydrogenotrophs have not evolved the metabolic versatility of the methylotrophs. The latter organisms use acetate by oxidizing the carbonyl to provide electrons for the reduction of the methyl to methane and they use the methyl compounds by disproportionation where some of the substrate is oxidized and some is reduced to methane. However, in hydrogenotrophs, the stoichiometric coupling of the methane-producing step to the recruitment of $CO_2$ into the pathway prohibits the input of additional intermediates. Furthermore, in the case of acetate, only one pair of electrons is available from the oxidation of the carbonyl, yet electron bifurcation requires that two pairs of electrons must feed into the hetero disulfide reductase complex. Although many hydrogenotrophic methanogens contain acetyl coenzyme A (acetyl-CoA) synthase/CO dehydrogenase (ACS/CODH), the essential enzyme for aceticlastic methanogenesis, they appear to use it only anabolically for $CO_2$ fixation and not catabolically for acetate utilization (Thauer, 2012).

# CONCLUSIONS

In the present study we determined the diversity of methanogens along the hindgut of horse and donkey by methods of molecular biology, using the archaeal 16S rRNA gene as marker and the following conclusions were derived from the obtained results.

A relatively high diversity of methanogens was found in the horse and donkey hindgut by DGGE and RFLP analyses of the archaeal 16S rDNA fragments.

The PCR-DGGE study, performed to obtain a preliminary insight of the diversity of the methanogenic archaea in the samples from the six different parts of the horse and donkey digestive tracts, showed that the differences in the archaeal community distribution is in correlation with those represented in the phylogenetic analysis.

The RFLP analysis corresponded well with the phylogenetic analysis of the sequenced 16S rDNA fragments.

The phylogenetic analysis determined represenatives of four methanogenic orders in the horse hindgut and three methanogenic orders in the donkey hindgut. The diversity of the hindgut methanogens was much lower in the donkey compared to the horse.

The distribution of the methanogens along the equine hindgut was dependent on the part of the digestive tract and the incidence of different ribotypes was animal-dependent.

The Methanomicrobiales (*Methanocorpusculum-like*) clones represented 32% in the horse hindgut, they were present in all the hindgut segments, but dominated in the horse caecum.

The Methanomicrobiales (*Methanocorpusculum-like*) clones represented 65% in the donkey hindgut, they have not been detected in right and left ventral colon (DC1,DC2), but they dominated in the other segments of the donkey hindgut, i.e.caecum (DCe), left and right dorsal colon (DC3, DC4) and faeces (Dfe).

The Methanobacteriales (*Methanobrevibacter-like*) clones represented 38% in the horse hindgut, they have not been detected in the horse caecum, they were present in all the following hindgut segments, and they dominated in the horse left ventral colon (HC2), right dorsal colon (HC4) and faeces (HFe).

The Methanobacteriales (*Methanobrevibacter-like*) clones represented 28% in the donkey hindgut, they have not been detected in the donkey left and right dorsal colon (DC3, DC4), they were rare in the caecum and faeces, but absolutely they dominated in the donkey right and left ventral colon (DC1, DC2).

The Methanomassilicoccales clones represented 27% in the horse hindgut, they were present in all the parts of horse hindgut with the highest numbers in the left dorsal colon (HC3).

The Methanomassilicoccales clones represented 4% in the donkey hindgut and they were present in the caecum (DCe), left dorsal colon (DC3) and faeces (DFe).

The Methanosarcinales clones were detected in low number in the horse left and right dorsal colon (HC3, HC4) and faeces (HFe), but they have not been detected in the donkey hindgut.

The absence of *Methanobrevibacter* species and the dominance of *Methanocorpusculum* specie*s* in the caecum of both the animals reflect specific metabolic processes in this part of the digestive tract and indicate the possible differences in the metabolic activities among the hindgut segments of equidae.

The hydrogenotrophic methanogens were dominant in both the hindguts, which indicates that hydrogenotrophic methanogenesis is the major methane production pathway in the hindgut of both the animals.

The methylotrophic methanogens were detected in low numbers in both the hindguts, which indicates that methyamines are produced during the digestion of plant diet and can be used for the methane formation.

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