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Microbiome community of farmed Common eland (*Taurotragus oryx*) under varied forage quality regimes

MASTER'S THESIS

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Declaration

I hereby declare that I have done this thesis entitled “Microbiome community of farmed Common eland (*Taurotragus oryx*) under varied forage quality regimes” independently, all texts in this thesis are original, and all the sources have been quoted and acknowledged by means of complete references and according to Citation rules of the FTA.

In Prague date

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Lucie Benešová

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Abstract

Ruminants are one of the most successful groups of herbivorous mammals. Their evolutionary success is greatly influenced by the microbiome, which is helping them to degrade cellulose, as well as it serves as a direct source of microbial protein. The majority of research of ruminant gastrointestinal tract (GIT) microbiome is focused on the rumen microbes of cattle. But there is not so much known about the faecal microbiome, even if the collection of faecal samples is not invasive and could be used also for wild species of ruminants. The aim of this research was to identify faecal and rumen microbiome of farmed Common Eland (*Taurotragus oryx*), to detect whether the faecal and rumen microbiome varies between each other and to determine if there are differences in microbiome due to changing diets. The research was done on the University Farm Estate Lány in the Czech Republic. Six adult eland males were involved in 3 treatments. The first treatment was grass hay only diet, the second treatment was alfalfa silage diet and the third treatment (control) was a common daily diet composed by a mixture of corn silage, alfalfa hay, meadow grass hay and barley straw. There were collected fresh faecal samples, post-mortem rectum and rumen samples. The samples were analysed by molecular methods. Changes of faecal microbiome due to different diets were analysed using PCR-amplified 16S rRNA, primarily by DGGE (degradation gradient gel electrophoresis) with following sequencing of important bands. There were also done Next Generation Sequencing (NGS). There were major changes of microbiome due to varied diets as well as significant difference between rumen and faecal samples. In all faecal samples, the most abundant phyla were Firmicutes (58-70%), on the other hand the dominant phylum in rumen samples were Bacteroidetes (60.22%). Interesting difference between grass hay diet and alfalfa silage was in the phylum Proteobacteria, which were in the alfalfa diet represented only by 0.65%, but in the grass hay diet, their abundance was 17.62%.

Key words: microbiome, antelope, large animal husbandry, ruminant physiology, nutrition, diet quality.

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1. Introduction and Literature Review

1.1. Microorganism

The microorganism is described as an organism (such as a bacterium or protozoan) of microscopic or ultramicroscopic size **(Merriam Webster, 2018).** Microorganisms are often illustrated using single-celled, or unicellular organisms; however, some unicellular protists are visible to the naked eye, and some multicellular species are microscopic.

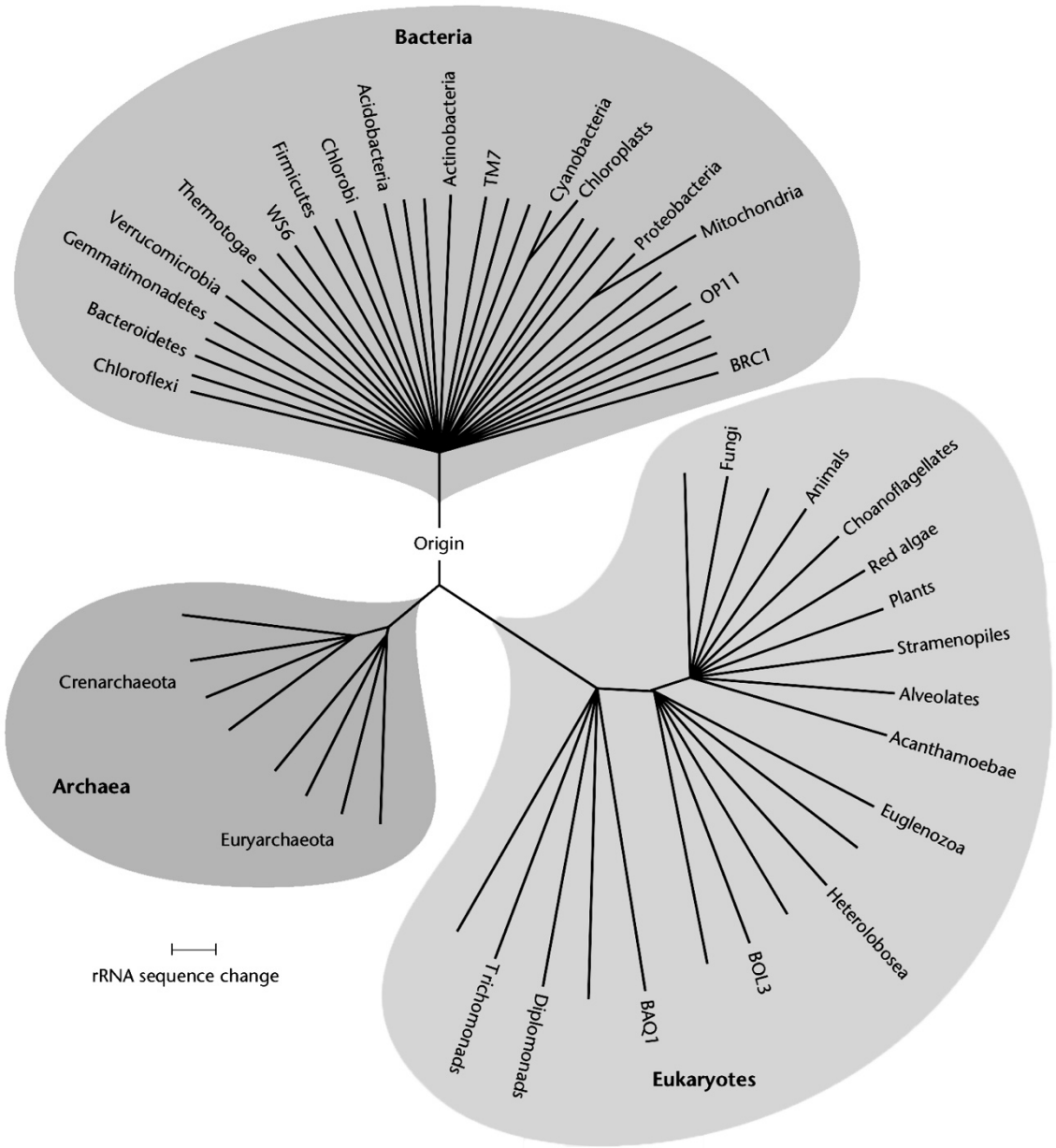

Figure 1: A molecular tree of life based on rRNA sequences (from Snyder et al. 2013).

According to the current view, all organisms on Earth belong to three major divisions called domains: the bacteria (formerly eubacteria), the archaea (formerly archaebacteria), and the eukaryotes **(Snyder et al. 2013).**

On the Figure 1. is the molecular tree of life based on rRNA sequences emphasizing the divergence of bacteria, archaea, and eukaryotes.

1.1.1.1. Bacteria

Bacteria is a major group of living organisms that are microscopic and mostly unicellular, with a relatively simple cell structure typically contained within a cell wall and lacking a cell nucleus (Cleveland & Morris 2015).

Bacteria are very small prokaryotic microorganisms, usually measuring 0.3 to 2.0 micrometres in diameter. Bacteria are the dominant living organisms on Earth, having been existing for possibly three-quarters of Earth history and having adapted to almost all available environmental habitats. They are found almost everywhere, being abundant in soil, water, and the digestive tracts of animals. Each species or group of bacteria is physiologically adapted to survive in one of the countless habitats created by various combinations of space, food, moisture, light, air, temperature, inhibitory substances, and other organisms in their environment. They exhibit exceptionally diverse metabolic abilities and can use almost any organic compound, and some inorganic compounds, as a food source. **(Hungate et al. 2016; Britannica Academic 2018).**

Although bacterial cells are much smaller and simpler in structure than eukaryotic cells, the bacteria are an extremely diverse group of organisms that differ in size, shape, habitat, and metabolism **(Britannica Academic 2018)**.

The morphology of bacteria can be distinguished microscopically and delivers the basis for classifying the bacteria into major groups. Three main shapes of bacteria are: spherical (coccus), rod (bacillus), and twisted rod (spirillum) **(Hungate et al. 2016)**. These and also some other common shapes (morphology) of bacteria are shown in the Figure 2.

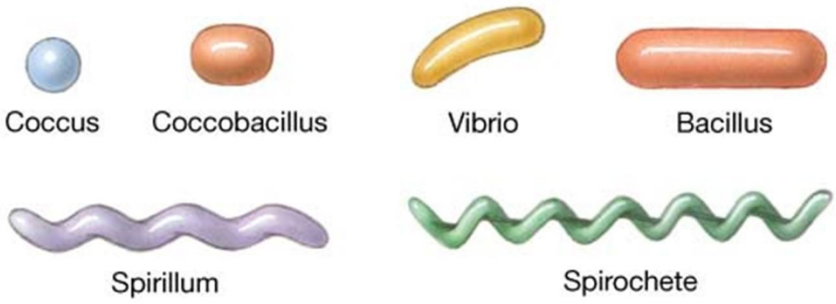

Figure 2: Common shapes of bacteria. Source: Microbeonline.com

Bacteria are divided based on a laboratory staining test that has been in use since 1884. It was developed by Danish bacteriologist Hans Christian Gram (1853–1938), in the laboratory of the famous Paul Ehrlich (1854–1915) in Berlin. The test combines two dyes, one is violet and the other purple. Bacteria are fixed on a glass plate by briefly running it through a flame. The violet solution is added first, second is added the purple one, each step is followed by a few washes. This procedure makes the bacteria look either bright red-violet or dark purple (visible under a microscope). The red-violet bacteria are called Gram negative, and the dark purple ones are called Gram positive. The test is simple, fast, and inexpensive. The difference in color is due to the different membrane. Although all eubacteria have membranes of similar lipid composition, Gram-negative bacteria are surrounded by two membranes, whereas Gram-positive bacteria have only one (and thick structure mainly consisting of a polymer called peptidoglycan). However, the difference between these groups seems to be more fundamental than the possession of an outer membrane **(Snyder et al. 2013, Wassenaar 2011).**

Despite all the research which have been done, most of the bacteria have not been characterized and the vast majority of the microbial world comprises from uncultured organisms **(Nocker et al. 2007; Rappé & Giovannoni 2003).**

1.1.1.1.1 Bacteroidetes

The phylum *Bacteroidetes* is a very diverse bacterial phylum, the name of this group changed several times over the past years. It is also known as the *Cytophaga–Flexibacter–Bacteroides* (CFB) group, an appellation that reflects the diversity of organisms found in this phylogenetic group (**Woese 1987; Gupta 2004; Thomas et al. 2011**).

Bacteroidetes are the major members of the microbiota of animals, particularly in the digestive tract, can act as pathogens and are often found in soils, oceans and freshwater. In these divergent ecological niches, Bacteroidetes are increasingly

considered as specialists for the degradation of high molecular weight organic matter, for example proteins and carbohydrates (**Thomas et al. 2011)**.

According to the Bergey's Manual of Systematic Bacteriology **(Bergey 1923),** the *Bacteroidetes* phylum comprises four classes: *Bacteroidia*, *Flavobacteria*, *Sphingobacteria*, and *Cytophagia*, representing around 7000 different species (NCBI, October 2010). While the digestive tract microbiota is mainly composed of species from the *Bacteroidia* class, environmental *Bacteroidetes* belong primarily to the *Flavobacteria*, *Cytophagia*, and *Sphingobacteria* classes (**Thomas et al. 2011**).

Uncultured and therefore uncharacterized Bacteroidetes lineages are abundant in many natural environments which specialize in lignocellulose degradation. However, their metabolic role remains mysterious, as well-studied cultured Bacteroidetes have been shown to degrade only soluble polysaccharides within the human distal gut and herbivore rumen (**Naas** et al. 2014).

Bacteroidetes are part of common, healthy mammal microbiome. They are the most studied, involving the genus Bacteroides (they are an abundant group in the faeces of endothermic animals), and genus Porphyromonas, that are colonizing the human oral cavity. The class Bacteroidia was previously named Bacteroidetes, the name was changed in the fourth volume of Bergey's Manual of Systematic Bacteriology **(Gupta 2004).**

1.1.1.1.2 Proteobacteria

Proteobacteria comprise the largest and most diverse phylum of bacteria, and they have widespread phylogenetic, ecological and pathogenic importance. All Proteobacteria are Gram-negative, with a lipopolysaccharide-containing outer membrane. Therein ends the similarity as the bacteria within this phylum show considerable differences in morphology, motility and metabolism. Rods and cocci, curved, spiral, ring-shaped, filamentous and sheathed bacteria have all been described (**Mukhopadhya** et al. 2012**).** They include a wide variety of pathogens, such as *Escherichia, Salmonella, Vibrio, Helicobacter, Yersinia, Legionellales*, and many other notable genera. Others are free-living (non-parasitic), and include many of the bacteria responsible for nitrogen fixation (**Wu et al**. 2009).

The Proteobacteria phylum is so big that it has been subdivided into five subphyla: Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria, Epsilonproteobacteria (**Wassenaar 2011; Mukhopadhya** et al. 2012**).**

The Alphaproteobacteria comprise mainly phototrophs, chemolithotrophs and chemoorganotrophs. Some of these bacteria are plant symbionts whereas others belonging to the *Rickettsiaceae*, *Brucellaceae* and *Bartonellaceae* family are putative human pathogens. The mitochondria of eukaryotes are believed to be derived from *Rickettsia spp*., forming the basis for the 'endosymbiotic hypothesis' that certain organelles originated as free-living bacteria taken inside eukaryotic cells as endosymbionts during early evolution (**Martin** & **Müller** 1998; **Mukhopadhya** et al. 2012).

Betaproteobacteria comprise several aerobic and facultative bacteria that have a key role in nitrogen fixation in soil. Pathogenic species include the *Neisseriaceae* and *Burkholderiaceae* families.

Gammaproteobacteria are the largest group of Proteobacteria; medically important pathogens belonging to the Enterobacteriaceae, Vibrionaceae and Pseudomonadaceae families are part of this group.

Deltaproteobacteria have two main branches: aerobic and fruiting-body-forming bac- teria (Myxobacteria), and obligate anaerobic bacteria that are sulfate-reducing and sulfur-reducing. Epsilonproteobacteria have many species that inhabit the human gastrointestinal tract, including Helicobacter spp., Campylobacter spp. and Wolinella spp. Several other members have been isolated from cold and hydro- thermal environments. Lastly, Zetaproteobacteria are a recent addition composed of a sole member, Mariprofundus ferrooxydans **(Mukhopadhya** et al. 2012).

1.1.1.1.3 Firmicutes

The Firmicutes are mostly Gram-positive bacteria, but some of them have a porous pseudo-outer membrane (they are Gram-negative), for example *Zymophilus*, *Pectinatus*, *Megasphaera* and *Selenomonas*. They have often rounded, cocci shape or rod-like shape – for example *Bacillus* spp. The considerable part of them creates very resistant endospores, which helps them to survive in harsh conditions. Because of this ability, they are living in a diverse range of environments.

The phylum Firmicutes is often divided into three groups – parasitical *Mollicutes*, anaerobic *Clostridia* and *Bacilli* which are facultatively or obligately anaerobic. Some of Firmicutes are also pathogenic and some are also found in the intestine, and it is claimed to have an effect on obesity **(Wolf et al. 2004; Ley et al. 2006).**

A lot of species of Firmicutes have been traditionally used for fermentation of food and dairy products. Since these are known to be completely safe for consumption, they were chosen as top candidates to function as a probiotic: species belonging to *Lactococcus*, *Lactobacillus*, *Enterococcus Bifidobacterium* (Actinobacteria), and a couple of other genera **(Wassenaar 2011).**

1.1.1.2. Archaea

Archaea look like bacteria, live like bacteria, and for a long time were thought to be bacteria (and some microbiologists still support this view) **(Wassenaar 2011).**

Archaea and Bacteria have traits in common – both of them have circular DNA and both groups lack intracellular organelles. But based on the genomic studies, chemistry and physiology they are considerably distinct **(Britannica Academic 2018)**.

The name archaea imply that they are very old (the Greek "arkhaios" means ancient), but we do not know whether they existed before bacteria, arrived later, or developed simultaneously **(Wassenaar 2011)**.

The archaea are a very diverse group of organisms and are sometimes divided into two phyla, the Euryarchaeota, containing methanogens, halophiles, and hyperthermophiles, and the Crenarchaeota, containing sulfur-dependent thermophiles. While substantial progress in research on archaea is being made, much less is known about the archaea than about the bacteria, although many of the components of the replication, transcription, and translation systems of archaea more closely resemble those of eukaryotes than they do those of bacteria **(Snyder et al. 2013).**

1.1.1.3. Protozoa

Protozoa is an informal term for single-celled eukaryotic organisms, either free-living or parasitic, which feed on organic matter such as other microorganisms or organic tissues and debris. Historically, the Protozoa were classified as "unicellular animals", as distinct from the Protophyta, single-celled photosynthetic organisms (algae) which were considered primitive plants. Modern ultrastructural, biochemical, and genetic techniques have shown that protozoa, as traditionally defined, belong to widely divergent lineages and only the ciliates (*Ciliophora*) formed a natural group, or monophyletic clade (**Adl et al. 2012**).

Most of the protozoa found in the rumen are ciliates (phylum Ciliophora). Many ciliates use cilia (tail-like structures) to move around, and to move food particles into their mouths. Most ciliates do not live inside another organism, but some exist in the rumen, such as Entodinium. In the rumen, two types of ciliates are found; the holotrichs and the spirotrichs. The holotrichs convert soluble sugars into starch, while spirotrichs consume starch and cellulose **(Hungate 1975**).

1.1.1.4. Fungi

Fungi is any member of the group of eukaryotic organisms that includes microorganisms such as yeasts and molds, as well as the more familiar mushrooms. These organisms are classified as a kingdom Fungi. Analyses using molecular phylogenetics support a monophyletic origin of this group. The taxonomy of the Fungi is in a state of constant flux, especially due to recent research based on DNA comparisons (**Hibbett et al. 2007)**.

The fungi in the rumen are anaerobes. Rumen fungi have been shown to digest cellulose and xylans, which shows that they may play a role in helping the ruminant host to digest plant matter (**Science on the farm 2018**).

1.2. Microbiome

Microbiome is defined as a community of microorganisms (such as bacteria, fungi, and viruses) that inhabit a particular environment and especially the collection of microorganisms living in or on the human/animal body (**Merriam Webster, 2018**). There is also often used a synonymous term "microbiota" which refers to ecological community of commensal, symbiotic and pathogenic microorganisms (**Lederberg & McCray 2001; Thursby & Juge 2017**).

1.2.1. Animal microbiome

Microorganisms formed spatially organized communities as long as 3.25 billion years ago, and some of them left relics recorded in the fossil findings (**Allwood et al. 2006**). Multicellular eukaryotes have existed for 1.2 billion years. The long period of interactions between multicellular life-forms and microbial communities preceded, and probably influence the evolution of vertebrates itself. Although the potential for

pathogenic interactions exists, mammals have evolved to form symbiotic relationships with a variety of microbes, where mutualism provides numerous nutritional, developmental, and physiological benefits **(Walter et Ley 2011; Taschuk & Griebel 2012).**

The composition of the vertebrate gut microbiota is influenced by diet, host morphology and phylogeny (**Ley et al. 2008; Thursby & Juge 2017**). On the other hand, **Turnbaugh et al. (2006)** has directly demonstrated that the composition of gut microbial communities can alter host metabolism (**Koren et al. 2012; Vijay-Kumar et al. 2010**)

Davies (2001) suggest that although completing the human genome sequence was a "crowning achievement" in biology, it would be incomplete until the synergistic activities between humans and microbes living in and on them are understood.

1.2.1.1. Ruminants

Ruminants are one of the most successful groups of herbivorous mammals on the planet, with around 200 species represented by approximately 75 million wild and 3.5 billion domesticated individuals worldwide **(Hackmann 2010; Clauss & Hofmann 2014; Henderson et al. 2015).** The success of wild ruminants can be largely explained by their ability to digest fibrous plant materials **(Hungate 1966).** Ruminants themselves do not produce fibre-degrading enzymes. But they were evolving together with microorganisms for millions of years, and the rumen was inhabited by diverse and interdependent populations of bacteria, protozoa, and fungi which are helping ruminants to break up and digest cellulose. These rumen microbes ferment feed to form volatile fatty acids that are major nutrient sources for the host animal and contribute significantly to ruminant productivity. The host also uses microbial biomass and some unfermented feed components once these exit the rumen to the remainder of the digestive tract **(Russell & Rychlik 2001; Clauss & Hofmann 2014; Henderson et al. 2015).**

Within this microbiome, bacteria are the dominant domain and make the greatest contribution to digestion and conversion of feeds to volatile fatty acids and microbial proteins **(Kim et al. 2011; Liu et al. 2016; Saengkerdsub & Ricke 2013; Henderson et al. 2015).**

The majority of research of the microbial community of ruminants has been focused on livestock members of the Bovidae (cattle), while there is not so much known about the microbiome of wild ruminants (**Roggenbuck** et al. 2014).

1.2.1.1.1 Rumen microbiome

Although all rumen samples in the study of **Henderson et al. (2015)** were obtained from a wide range of ruminant species, diets, and geographical locations, the 30 most abundant bacterial groups were all found in over 90% of samples, and together formed 89.4% of all sequence data. The 7 most dominant bacteria species inhabiting rumen were *Prevotella*, *Butyrivibrio*, and *Ruminococcus*, as well as unclassified *Lachnospiraceae*, *Ruminococcaceae*, *Bacteroidales*, and *Clostridiales* (67.1% of all bacterial sequence data). These species could be described as "core bacterial microbiome" at the genus level or higher because they were found in a considerable selection of ruminants. But these bacteria were not equally abundant in all animal species. This research (**Henderson et al. 2015)** also showed that only 14% of bacteria fell within a named species, and 70% were not even within a recognised genus.

Like other herbivores, the giraffe (*Giraffa camelopardalis*; Linnaeus 1758) depends on symbiosis with microorganisms in the digestive system to utilize cellulose and hemicellulose. In the study of **Roggenbuck** et al. (2014) a total number of 21 phyla were observed for all samples combined. The majority were represented by Firmicutes (50% - relative abundance) and Bacteroidetes (30%), then there were present Proteobacteria (4%), Cyanobacteria (1%), Actinobacteria (1%) and Euryarchaeota (2%). The phyla of Acidobacteria, Chloroflexi, Crenarchaeota, Fibrobacteres, Fusobacteria, Gemmatimonadetes, Nitrospira, Planctomycetes, Spirochetes, Synergistetes, Tenericutes and Verrucomicrobia, were detected to each constitute below 1%.

On the phylogenetic family level only 53% of the sequences were assigned to known taxa of *Ruminococcaceae* (21%), *Lachnospiraceae* (11%), *Prevotellaceae* (10%), *Veillonellaceae* (7%), *Methanobacteriaceae* (2%), *Porphyromonadaceae* (1%) and *Streptophyta* (1%). Only 28% of all sequences were assigned to genus level. The most abundant genera (identified), independent of ruminal fraction, were *Prevotella* (6%), *Succiniclasticum* (5%), *Oscillibacter* (4%), *Methanobrevibacter* (2%), *Ruminococcus* (1%), *Barnesiella* (1%) and *Pseudobutyrivibrio* (1%). All archaeal sequences belonged to the family Methanobacteriaceae, specifically to the genera *Methanobrevibacter* (98% of the sequences) and *Methanosphera* (**Roggenbuck** et al. 2014).

1.2.1.1.2 Faecal microbiome

According to **Drasar & Barrow (1985**) microbiome of the lower gastrointestinal tract of livestock (*Bos taurus*; Linnaeus 1758) is predominantly occupied by strict anaerobes, for example, *Clostridium* spp., *Bifidobacterium* spp. and *Bacteroides* spp., on the contrary, facultative anaerobes, are occurring in at least 100 times lower numbers. Similar results were reported in the study of the faecal microbiome of dairy cows by Dowd et al. (2008) in which the prevalent genera found were *Lachnospira*, *Akkermansia*, *Porphyromonas*, *Alistipes*, *Lachnospiraceae*, *Bacteroidales*, *Prevotella*, *Clostridium*, *Ruminococcus*, *Bacteroides* and *Enterococcus* spp. *Clostridium* spp. Was found in all livestock samples in the study (**Dowd** et al. 2008) and it formed about 20 % of the total microbial populations. *Clostridium* is an extensive genus abundant in the digestive tract. It could have either negative either positive effects on the host animal. The influence on the animal often depends on the specific species of *Clostridium* (Grizard & Barthomeuf 1999; Kanauchi et al. 2005). The negative effects are known for example in the species *Clostridium botulinum*, *C. difficile* and C. *tetani* (Dowd et al. 2008; Attwood et al. 2006; Songer 2004). But there are also species of *Clostridium*, which could help with the digestion of high-fibre roughage and some of them are considered as probiotics (**Widyastuti et al. 1992)**.

Escherichia coli is easily cultured and ubiquitous in the faeces of animals, so that they are often used as a marker of fecal contamination in water supplies, however they typically comprise less than 1 % of the intestinal bacterial populations (**Drasar & Barrow 1985).**

In the research of (**Rudi** et al. 2012) Firmicutes dominated the microbiota of cattle in the samples analyzed with 81.9% of all the reads, followed by Proteobacteria (9.6%), Bacteroidetes (5.4%), and Actinobcateria (2.8%).

Sheep (*Ovis aries;* Linnaeus 1758) faecal microbiome was made over 80% by Firmicutes and Bacteroidetes. And Firmicutes and Bacteroidetes made over 80% of total bacteria in all cases he archaeal Euryarchaeota was the fifth, seventh and third most abundant phylum (**Tanca et al.** 2017).

1.2.1.2. Changes of ruminant microbiome due to different diets

1.2.1.2.1 Rumen

The structure and function of the microbiome inhabiting the rumen of cattle (*Bos taurus*; Linnaeus 1758) are, amongst other factors, mainly shaped by the animal's feed intake. Samples were obtained from three fistulated Jersey cows rotationally fed with corn silage, grass silage or grass hay, each supplemented with a concentrate mixture **(Deusch et al. 2017).**

Unclassified *Clostridiales* were most abundant in bovines fed forage and least abundant in bovines fed high concentrate diets. In caprids, cervids, and camelids these diet differences were far less pronounced. *Butyrivibrio* was most abundant in rumen samples from bovines fed mixes of forage and concentrates. *Fibrobacter* was most abundant in bovines fed forage. When concentrate was included in cattle diets, the relative abundance of *Fibrobacter* was lower, but it was still more abundant than in other animals (**Henderson et al. 2015).**

In the giraffes, there were not significant changes in microbiome on phyla level between the different diet groups (**Roggenbuck** et al. 2014).

In the study of **Kala et al.** (2017) of buffalo rumen microbiome the population density of total bacteria, *Fibrobacter succinogenes*, methanogens and fungi was similar in all the three diets, but the numbers of *Ruminococcus flavefaciens* and *Ruminococcus* albus were significantly lower ($P < 0.05$) in the rumen liquor of buffaloes fed on 100% total digestible nutrients diet as compared to the animals fed 70% total digestible nutrients diets. High fiber groups was positively correlated with higher *Ruminococcus* population. Even after feeding of similar diet, individual variation of animal plays important role in rumen microbiome of ruminant **(Kala et al.** 2017).

In the study of **Patel et al**. (2014) Indian cattle (Kankrej breed) was gradually adapted to a high-forage diet containing 50 % of forage (50 % forage: 50% concentrate), 75 % of forage and 100 % of forage. Taxonomic analysis indicated that rumen microbiome was dominated by Bacteroidetes followed by Firmicutes, Fibrobacter, Proteobacteria, and Tenericutes. ratios of the phyla Firmicutes/Bacteroidetes were compared during three diet treatments. Ratio of Firmicutes/Bacteroidetes was found to be decreasing from 50% to 100% treatment.

1.2.1.2.2 Faecal

In the research of **Shanks** et al. (2011) the bacterial community composition of cattle correlated significantly with fecal starch concentrations, largely reflected in changes in the Bacteroidetes, Proteobacteria, and Firmicutes populations. Results demonstrate that Firmicutes (55.2%) and Bacteroidetes (25.4%) are the most abundant phyla, then there were present Tenericutes (2.9%) and Proteobacteria (2.5%) Other phyla represented were the Actinobacteria (0.73%), Spirochaetes (0.54%), Verrucomicrobia (0.19%), Cyanobacteria (0.15%), Fibrobacteres (0.02%), and Lentisphaerae (0.02%). Percentages of abundance of Firmicutes and Bacteroidetes change dramatically from the forage and processed-grain groups to the unprocessed-grain group. Relative abundance of Bacteroidetes increases with the fecal starch concentration and the abundance of Firmicutes decreases with increasing fecal starch concentrations (**Shanks** et al. 2011).

1.3. Description of model species

1.3.1. Common Eland

In the genus *Taurotragus*, there are two species – The Giant eland (*Taurotragus derbianus*; Gray 1847) and The Common eland (*Taurotragus oryx*; Pallas 1766). The genus Taurotragus is sometimes due to molecular and chromosomal studies studies (**e.g. Fernández & Vrba 2005**) subsumed within genus *Tragelaphus* **(Encyclopedia of Life 2018)**.

There are three subspecies of common eland. First subspecies is Livingstone's Eland (*Taurotragus oryx livingstonei*) found in Central woodlands. They are brown with up to 12 stripes. Second subspecies is Cape Eland (*Taurotragus oryx oryx*) which live in South and South West Africa. They have tawny colour and adults lose their stripes. The third subspecies is East African Eland (*Taurotragus oryx pattersonianus*) they are inhabiting East Africa. They have a rufous tinge with up to 12 stripes **(Castelló 2016)**.

The Derby eland (*Taurotragus derbianus*) is the largest antelope, however The Common eland is the second largest (**Underwood 1979**). The range of shoulder height is between 152 and 183 cm in males and from 125 to 153 in females. The weight of males ranges from 450 to 940, but the average is 500 – 600 kg. Females are smaller, so the

weight ranges between 320 and 470 kg with the average weight 340 – 445 kg (**Estes 1991; Pappas 2002**).

Common Eland formerly occurred in the savannah woodlands on the east and south of Africa, spreading into high-altitude grasslands and the arid savannahs and scrublands of the Kalahari and Karoo in southern Africa. Its range shrink to more than half of its original range due to the increase and expansion of human population, excessive hunting and habitat loss. Their numbers have declined radically since the 1970s as a result of civil wars and their aftermath in countries such as Uganda, Rwanda, Angola and Mozambique. They have been reintroduced to areas of southern Africa (especially South Africa) and introduced outside of their natural range to southern and central Namibia **(Kingdon 1997; IUCN SSC Antelope Specialist Group 2016).**

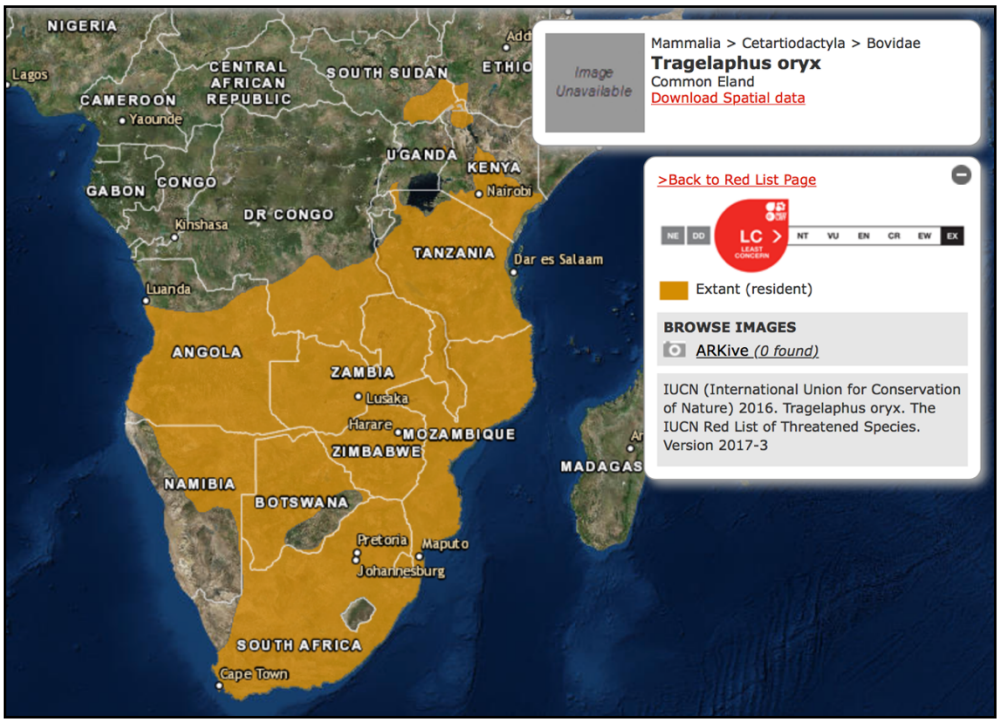

Figure 3: Distribution of Common Eland (Taurotragus oryx) according to The IUCN Red List of Threatened Species (2017)

The animals inhabit open plains, savannahs, and lightly wooded areas and avoid thick forests (**Hosking & Withers 1996**). They might occupy altitudes up to 4 600 m (**Estes 1993**). Trees and shrubs commonly occurring in the eland's diet are *Acacia*, *Combretum*, *Commiphora*, *Diospyros*, *Grewia*, *Rhus*, and *Ziziphus*. Elands also feed on forbs (non-woody dicotyledons) from the family *Compositae*, including *Acanthospermum*, *Bidens*, *Tagestes*, and *Tarchonanthus*, and fruits from *Securinega* and

Strychnos. Prevailing grasses are *Setaria* and *Themeda* (**Hillman 1979; Kingdon 1997; Skinner & Chimimba 2005)**.

Elands have been categorised as intermediate feeders, preferring forbs along with foliage of shrubs and trees (**Hofmann & Stewart 1972**), and as browsers that have adapted to grazing **(Buys 1990**). Eland could graze during the wet season, when grasses are abundant, but browse more during dry winter season. Even though eland drink water, when it is enough of it, they acquire most of their water from their diet (**Buys 1990; Skinner & Chimimba 2005**). They are crepuscular and feed during sunrise and dusk. The main reason (80 %) of the variability in daily feeding times are environmental factors (**Lewis 1978**).

Eland can be raised for meat and dairy production. They are easily tamed and can be fully domesticated. In general, they have a mild temperament and are quite calm. However, they require a large area in which to graze. In South Africa, they are domesticated for meat and dairy production. Eland milk has nearly thrice more of the fat and two times more of the protein than the milk from a dairy cow (**Pappas 2002; Kingdon 1997; Lightfoot & Posselt 1977; Hansen et al. 1985**).

1.4. Molecular Methods for Microbial Identification

Culture-based methods are extremely time-consuming and to date, we have only been able to culture approximately 1 % of the bacteria from the digestive tract because almost all of these bacteria will die if they are exposed to oxygen (they are obligatory anaerobic) (**Hungate 1975**). These are the reasons for the wide use of molecular methods.

Molecular biological techniques offer new opportunities for the analysis of the structure and species composition of microbial communities. Genetic fingerprinting techniques provide a pattern or profile of the genetic diversity in a microbial community **(Muyzer et al. 1993; Muyzer & Smalla 1998).**

1.4.1. DNA isolation

DNA isolation is a fundamental initial step for molecular genetic studies.
Most of the automated extraction procedure consists of multiple steps: lysis of cells and/or pathogens, inactivation of cellular nucleases, capture of nucleic acids, purification, and

elution of the purified nucleic acids. DNA and/or RNA can be isolated in one step through binding to the silica surface of magnetic particles in the presence of a chaotropic salt.

1.4.2. Polymerase Chain Reaction

The Polymerase Chain reaction (PCR) is defined as the in vitro enzymatic synthesis and amplification of specific DNA sequences. This extremely sensitive technique allows the amplification of genes and other regions of DNA, facilitating their cloning and study **(Templeton 1992; Snyder et al. 2013).**

In 1983 Mr. Kary Mullis invented and implemented a polymerase chain reaction (PCR). This invention was so significant for scientific work that he received the Nobel Prize in 1993 (**Gupta et al. 2016**). Although Millis is widely credited with the original invention of PCR, the success application of PCR as we know it today required considerably further development by his colleagues at Cetus Corp, including colleagues in Henry Erlich's lab. The DNA polymerase originally used for the PCR was extracted from the bacterium *Escherichia coli.* Although it had been a valuable tool, it had distinct disadvantages. For PCR, the reaction must be heated to denaturate DNA after each round of synthesis. Unfortunately heating inactivated *E. coli* DNA polymerase, so the enzyme had to be added at the start of each cycle. The solution was found after timely isolation of thermostable polymerase enzyme from a thermophilic bacterium *Thermus aquaticus*, isolated from thermal springs. Another major step of improving PCR was development of first thermocycler, which makes whole process significantly faster (**Bartlett & Stirling 2003**; **Snyder et al. 2013**).

Like DNA replication in an organism, PCR requires a DNA polymerase enzyme that makes new strands of DNA, using existing strands as templates. The DNA polymerase typically used in PCR is called Taq polymerase, after the heat-tolerant bacterium from which it was isolated (***T****hermus* ***aq****uaticus*). The polymerase chain reaction is based on synthesizing new DNA strands complemental to the original template strand. There needs to be primer to add the first nucleotide, because polymerase can synthetize only on already existing 3'-OH group. This requirement makes it possible to delineate a specific region of template DNA that is needed to amplify. Finally, there will be billion amplicons of the specific DNA sequence (**Bartlett & Stirling 2003)**.

The basic steps of PCR are:

1. Denaturation (96°C): Heat the reaction strongly to separate, or denature, the DNA strands. This provides single-stranded template for the next step.
2. Annealing (55 - 65°C): Cool the reaction so the primers can bind to their complementary sequences on the single-stranded template DNA.
3. Extension (72°C): Raise the reaction temperatures so Taq polymerase extends the primers, synthesizing new strands of DNA (**Khan Academy 2018).**

1.4.3. Gel electrophoresis

The length and purity of DNA molecules can be accurately determined by the gel electrophoresis. Gel electrophoresis is a technique in which fragments of DNA are pulled through a gel matrix by an electric current, and it separates DNA fragments according to size. Each nucleotide in a nucleic acid molecule carries a single negative charge, which makes molecules to move uniformly toward the positive electrode. For DNA fragments, less than 500 nucleotides long, specially designed polyacrylamide gels allow separation of molecules that differ in length by as little as a single nucleotide. The pores in polyacrylamide gels, however, are too small to permit very large DNA molecules to pass; to separate these by size, the much more porous gels formed by dilute solutions of agarose (a polysaccharide isolated from seaweed) are used. DNA fragments of the same length form a "band" on the gel, which can be under UV light seen by eye if the gel is stained with a DNA-binding dye. These DNA separation methods are widely used for both analytical and preparative purposes **(Chaffey et al. 2003).**

1.4.4. Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) is based on electrophoresis of PCR-amplified 16S rRNA fragments in polyacrylamide gels containing a linearly increasing gradient of denaturants (formamide and urea). DNA fragments of the same length, but different sequences are separated according to their melting temperature. The melting of DNA fragments is a gradual process proceeding through so-called melting domains (stretches of base pairs with the same melting temperature). Once the melting domain with the lowest melting temperature reaches its melting temperature at a particular position in the DGGE gel, a transition of helical to partially melted molecules occurs, and migration of the molecule will practically halt. Sequence variation within

such domains causes their melting temperatures to differ. Sequence variants of particular fragments will, therefore, stop migrating at different positions in the denaturing gradient and hence can be separated effectively by DGGE. It is one of the most commonly used methods among the culture-independent fingerprinting techniques. This method is used to determine the genetic differences of microbial communities (**Muyzer** et al. 1993; **Munaut e**t al. 2011; **Zhou** & Li, 2015).

1.4.5. Sanger sequencing

Even though the original Sanger Sequencing technique (ddTTP - 2',3'-dideoxythymidine triphosphate) is outdated these days, this invention established the basis for modern sequencing technologies and allow scientist to sequence and study the genetic material of different organisms, including human. Sanger sequencing uses modified nucleotides (ddNTPs) to stop the replication process whenever a dummy nucleotide is incorporated. Dideoxynucleotide triphosphates (ddNTPs) lack the 3'-OH group of dNTPs that is essential for polymerase-mediated strand elongation in a PCR. Therefore, ddNTPs are used in combination with a modified Taq polymerase as 3'-end chain terminators in Sanger sequencing. Terminated replication occurs repeatedly, and the nucleic acid sequences of varying lengths, accumulated during the process, can be later used to determine the position of each nucleotide in the DNA sequence **(Estrada-Rivadeneyra 2017).**

During the Sanger sequencing the DNA sample is amplified, double stranded DNA is denatured using heat. Than a primer is annealed to the 5'end of DNA template, the primed DNA is equally divided into 4 reaction tubes along with DNA polymerase and all four dNTPs. Specially designed ddNTPs are added to different reaction tubes, only one type of ddNTP (A, T, G or C) is added to each reaction tube. The DNA polymerase incorporates the dNTPs normally to the primed DNA template until a ddNTP base is paired. Once the ddNTP base is paired the elongation process is terminated due to the lack of a 3'hydroxyl group on the ddNTP. This result in the formation of fragments with different lengths. The contents of each tube are then transferred into different lanes of a polyacrylamide gel and then subjected to gel electrophoresis. This result in the formation of band patterns across the gel that can be interpreted as the sequence of the DNA sample **(Estrada-Rivadeneyra 2017).**

1.4.6. Next Generation Sequencing (NGS)

Next generation sequencing (NGS), massively parallel or deep sequencing are related terms that describe a DNA sequencing technology which has revolutionised genomic research. Next-Generation Sequencing (NGS, or Next-Gen Sequencing) technologies are now making a greater impact on sequencing. The invention of the 454 sequencer in 2005 by Jonathan Rothberg represents a hallmark in modern sequencing technology. In fact, NGS is made of a number of robust technologies and, in general, is characterized by high speed and high yield (**Behjati & Tarpey 2013; Chiu 2015**).

Using NGS an entire human genome can be sequenced within a single day. In contrast, the previous Sanger sequencing technology, used to decipher the human genome, required over a decade to deliver the final draft. The main utility of NGS in microbiology is to replace conventional characterisation of pathogens by morph- ology, staining properties and metabolic criteria with a genomic definition of pathogens (**Behjati & Tarpey 2013**).

Sequencing without any prior selection of targets is known as shotgun sequencing and requires fragmentation of micrograms of DNA into short fragments of 50–500 base pairs. The alternative to shotgun sequencing is usually called targeted sequencing and involves an initial enrichment step that either amplifies the selected regions by PCR. Once the DNA has been prepared for either shotgun or capture sequencing, the fragments are used to generate a library. The library is constructed by ligating adapters to the fragments or by one or two PCR reactions where the PCR primers are tagged with sequences needed for the downstream reactions. The construction of the library is the critical step of the experimental design. The choice of barcodes dictates how many samples can be sequenced. The number of samples that can be analysed in the same experiment depends on a number of factors: 1) The number of available barcodes, 2) the sequencing capacity of the NGS platform, 3) the numbers and sizes of targeted regions and 4) the desired sequencing depth (**Børsting & Morling 2015).**

Next generation sequencing (NGS) technology has revolutionized genomic and genetic research. The pace of change in this area is rapid with three major new sequencing platforms having been released in 2011: Ion Torrent's PGM, Pacific Biosciences' RS and the Illumina MiSeq. (**Rothberg** et al. 2011; **Quail** et al. 2012)

Ion Torrent sequencing

The Ion Torrent PGM (semi-conductor technology) is detecting the protons released as nucleotides are incorporated during synthesis. The all-electronic detection system used by the ion chip simplifies and greatly reduces the cost of the sequencing instrument. The instrument has no optical components, and is comprised primarily of an electronic reader board to interface with the chip, a microprocessor for signal processing, and a fluidics system to control the flow of reagents over the chip. DNA is fragmented, ligated to adapters, and adaptor-ligated libraries are clonally amplified via emulsion PCR onto the surface of 3-micron diameter beads, known as Ion Sphere Particles (**Rothberg** et al. 2011; **Quail** et al. 2012; **Buermans & den Dunnen** 2014).

Template-bearing beads are enriched through a magnetic-bead-based process. Sequencing primers and DNA polymerase are then bound to the templates and pipetted into the chip's loading port. Individual beads are loaded into individual sensor wells by spinning the chip in a desktop centrifuge. The beads and the wells depth was designed to allow only a single bead to occupy a well. As sequencing proceeds, each of the four bases is introduced sequentially. If bases of that type are incorporated the protons are released. The release of the proton produces a shift in the pH of the surrounding solution proportional to the number of nucleotides incorporated in the flow. This is detected, proportional to the number of bases incorporated, by the sensor on the bottom of each well (**Rothberg** et al. 2011; **Quail** et al. 2012).

2. Aims of the Thesis

The aim of this thesis was to identify faecal and rumen microbiome of farmed Common Eland (*Taurotragus oryx*) for the first time. The second aim of this thesis was to detect whether the faecal and rumen microbiome varies between each other. The third aim was to determine if there are differences of microbiome due to the feeding of three different diets:

1) The mixture of Corn silage, meadow hay and lucerne (alfalfa) silage (common daily diet fed to elands on the farm);
2) The grass hay (Treatment A);
3) The lucerne (alfalfa) silage (Treatment B).

3. Methods

3.1. Data collection

The experiment was done at the University Farm Estate in Lány in the Czech Republic (Figure 4, 5, 6). On the farm, there were two breeding groups of common elands (*Taurotragus oryx*) – altogether approximately 50 animals. Animals were kept in the outside enclosure with possibility to hide in stable. During the cold season (from November till March) animals were held indoors (in the stable).

Figure 4: Enclosure of common elands in Lány

Figure 5: Elands in the stable in Lány

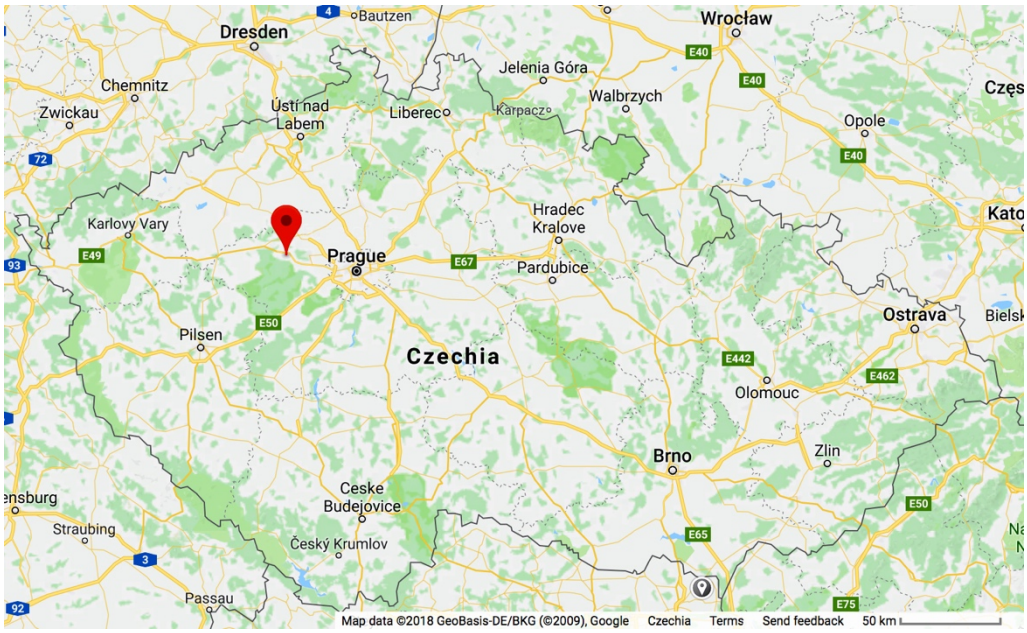

Figure 6: Location of the farm in Lány, Czech Republic (Google Maps. 2018).

3.2. Experiment design

Common eland (*Taurotragus oryx*) bred under the farm conditions, was chosen as a model species. Six individuals of the same sex, males, and age category, adult (i.e.> 3 years old), were involved in each treatment (Table 1). For the duration of the treatment animals were kept individually in separated boxes. Treatments consisted in feeding animals for 3 weeks on a monocot-only diet (grass/grass hay) and three weeks on dicot-only diet (lucerne silage). The third diet – common daily diet (fed also to the rest of the animals, which were not involved in any treatment) was a mixture of corn silage, alfalfa hay, meadow grass hay and barley straw. This diet served as control. The grass hay and alfalfa silage is shown on the Figure 7.

Figure 7: On the left picture, there is monocot-only grass hay and on the right picture, there is dicot-only lucerne silage.

First two weeks of treatment served for habituation of animals on different diet. Fresh faeces samples were collected during third week of treatment. Due to the nature of animals the direct collection of samples from rectum was impossible. Thus, samples were collected from the ground of boxes as fresh as possible. The Figure 8 shows fresh faecal samples before collection. There were also collected anonymous samples from animals in the breeding group, as well as samples from individuals included in the experiment, which were not separated at that time (control samples). There were also collected post-mortem rumen and faecal samples (from rectum) from the culled animals. Samples were stored in

Figure 8: Fresh samples before collection.

freezer. Collection of samples ran from May 2017 till November 2017. A list of each animal and the sample numbers of each are given in Table 1. The samples collection was done by Lucie Stoklasová, Pavla Hejcmanová, Petr Beluš and Lucie Benešová.

Table 1: List of animals and sample identification.

animal	Grass hay	Alfalfa hay	Control	† Rumen	† Faeces
180	S1	V1	K1A	B1	F1
181	S2; S2B	V2	K2A; K2B		
182	S3	V3	K3A; K3B; K3C	B3	F3
179	S4	V4	K4A; K4B	B4	F4
168		V5	K5	B5	F5
Hanno		V6A; V6B	K6. K6B. K6P	B6	F6
Daen			K9		
117			K10		
197				KB	KF
anonym			K11		
anonym			K12		
anonym			K13		
anonym			K14		

3.3. Analyses of samples

3.3.1. PCR methods

PCR analyses were performed in facilities of the Institute of Animal Physiology and Genetics of Czech Academy of Sciences, except Sanger sequencing which was performed by company SEQme s.r.o. (Dobříš, Czech Republic).

3.3.1.1. DNA isolation

DNA isolation was done by using commercial kit and procedures (PowerFecal® DNA Isolation Kit, MO BIO Laboratories a Qiagen company). The PowerFecal® DNA Isolation Kit is designed for fast and easy purification of both microbial and host genomic DNA from stool and feces (PowerFecal®, Qiagen).

The first step of isolation is the breaking of all cells (mechanically, thermally, chemically), which leads to the release of the cell content into the solution. Approximately 0.1 g of sample was weighted into Dry Bead Tube, containing garnet beads. The 750 µl of Bead Solution and 60 µl of C1 solution was added into each sample.

There was used modified procedure that uses FastPrep bead-beating equipment

for mechanical lyses of samples, which yields significantly higher DNA quality and concentration. Samples were vortexed for 30 seconds, heated up on 65 ℃ for 5 minutes, then vortexed for another 30 seconds and heated up on 65 ℃ for 5 minutes again. The samples were centrifuged at 11000 spins for 1 minute, supernatant was transferred into clean collection tube (2ml).

In the next step, nucleic acids are separated from other undesirable material (proteins, carbohydrates, lipids, cell wall remnants etc.) through filter columns. The 250 µl of C2 solution was added, briefly vortexed and incubated at 4 ℃ for 5 minutes. The samples were centrifuged at 11000 spins for 1 minute, the 600 µl of supernatant was transferred into clean collection tube (2ml). The 200 µl of C3 solution was added, vortexed briefly and incubated at 4 ℃ for 5 minutes. The samples were centrifuged at 11000 spins for 1 minute. All the following steps were performed according to the PowerFecal® DNA Isolation Kit Instruction Manual, which you can find in the appendix. In the last step, nucleic acids are released from the filter by water.

Obtained DNA was used to for all following analyses. Quality of obtained DNA was tested by microvolume spectrophotometer (NanoDrop OneC, Thermo Fisher Scientific Inc.). The Table 2. is describing Nucleic Acid (ng/uL) concentration of all samples. DNA sample dilution (Due to concentration of samples obtained from NanoDrop)

The samples were diluted according to concentration of Nucleic Acid (ng/uL) obtained from NanoDrop. The samples with concentration higher than 20 ng/uL were diluted in the ratio 1:5 with nuclease-free water (1 part sample, 5 parts water).

Table 2: Sample weight (m), concentration, PCR label and dilution.

sample code	**m (g)**	**Conc. of Nucleic Acid (ng/uL)**	**PCR label**	**dilution** (water:sample)
S1	0.116	12.41	1A	1:1
S2	0.108	14.07	2A	1:1
S2B	0.103	10.71	3A	1:1
S3	0.096	7.82	4A	1:1
S4	0.105	14.93	5A	1:1
V1	0.105	12.22	6A	1:1
V2	0.120	9.21	7A	1:1
V3	0.101	11.03	8A	1:1
V4	0.114	7.25	9A	1:1
V5	0.111	11.10	10A	1:1
V6A	0.117	7.39	11A	1:1
V6B	0.111	37.70	12A	5:1
K1A	0.114	11.55	2B	1:1
K2A	0.105	11.23	3B	1:1
K2B	0.114	9.04	4B	1:1
K3A	0.116	6.08	5B	1:1
K3B	0.106	6.39	6B	1:1
K3C	0.113	7.47	7B	1:1
K4A	0.109	7.07	8B	1:1
K4B	0.113	8.65	9B	1:1
K5	0.107	8.88	10B	1:1
K6	0.099	4.21	11B	1:1
K6B	0.111	11.71	12B	1:1
KP6	0.106	18.78	1C	1:1
K9	0.103	6.45	3C	1:1
K10	0.109	9.04	4C	1:1
K11	0.101	15.73	5C	1:1
K12	0.110	10.82	6C	1:1
K13	0.109	8.71	7C	1:1
K14	0.111	11.76	8C	1:1
B1	0.116	9.63	9C	1:1
F1	0.100	65.67	10C	5:1
B3	0.122	4.32	11C	1:1
F3	0.106	11.13	12C	1:1
B4	0.112	10.67	1D	1:1
F4	0.104	10.04	2D	1:1
B5	0.113	10.91	3D	1:1
F5	0.102	7.69	4D	1:1
B6	0.126	52.47	5D	1:1
F6	0.121	90.64	6D	5:1
KB	0.102	64.59	7D	5:1
KF	0.108	76.79	8D	5:1

3.3.1.2. DNA purification

In the presence of high salt, DNA binds to silica particles. The bound DNA is then washed to remove impurities from the original sample, and the clean DNA is eluted in water or TE buffer (ThermoFisher Scientific, 2015).

DNA purification was done by using commercial kit and procedure "QIAquick® PCR Purification Kit" (Qiagen company). The protocol of this procedure is provided in Appendix 2 (QIAquick® PCR Purification Kit Protocol).

3.3.1.3. Polymerase Chain Reaction (PCR)

During the DNA isolation, there was obtained a total DNA from all microorganisms. For further work, we are only interested in the part that is used for the identification of microorganisms, which is 16S ribosomal RNA (16S rRNA) in the case of the 16S genes. The variable V3 region of 16S rRNA was enzymatically amplified in the PCR with primers to conserved regions of the 16S rRNA genes (**Muyzer** et al. 1993). Amplification of 16S rRNA of bacterial DNA was performed on Biometra TAdvanced thermocycler (Analytik Jena AG, Germany), using universal primers 338GC and RP534. The nucleotide sequences of the primers are as following:

338GC:

CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCGCCGCACTCCTA
CGGGAGGCAGCAG

534RP:

ATTACCGCGGCTGCTGG

The composition of PCR solution according to **Muyzer** et al. (1993) is described in the Table 3.

Table 3: Composition of PCR solution

DNA template	1 uL
Forward primer 10x 338GC	1 uL
Reverse primer 10x 534RP	1 uL
PCR mix	15 uL
dH_2O	12 uL
Total	30 uL

The PCR program was – First step 3 min at 94°C to denature the template which was followed by 35 cycles comprising of 1 min at 94°C, 30 s at 55°C, 1 min at 72°C, and the final primer extension step at 72°C for 10 min **(Muyzer et al. 1993**).

Amplification products were analysed by electrophoresis in 1.5% agarose gels and then by ethidium bromide staining.

3.3.1.4. Gel electrophoresis

Gel electrophoresis is a method for separation and analysis of macromolecules (DNA, RNA and proteins) and their fragments, based on their size and charge. Gel electrophoresis was used for evaluation of quality of samples obtained from PCR. The gel consisted of 1,5 % agarose dissolved in 0.5 x TBE buffer (1:10 dilution of the concentrated 5 x TBE stock solution). The electrophoresis was performed for 20 minutes at 90 V. The gel was then stained with ethidium bromide and visualized under UV light at GelDoc system (Volber Lourmat). Positive reaction contained DNA fragments with size 200 bp. The recipe of TBE buffer 5 x stock solution is provided in the Table 4.

Table 4: Composition of 5 x Stock solution of TBE buffer

54 g	Tris base [2-Amino-2-(hydroxymethyl)propane-1,3-diol]
27.5 g	Boric acid (H_3BO_3)
20 ml	0.5 M EDTA (Ethylenediaminetetraacetic acid)

3.3.1.5. Denaturing Gradient Gel Electrophoresis (DGGE)

Denaturing gradient gel electrophoresis is fingerprinting technique, that analyses bacterial or archeal 16S rRNA amplicons and separates presented DNA on the basis of primary structure. It gives a profile of most abundant microorganisms (usually 99.9 % of total numbers). Because of its low cost and high speed, we performed PCR-DGGE on all our samples for fast initial screening. The analysis was performed on D-Code Universal Mutation Detection System (BioRad) according to the manufacturers protocol. The

After electrophoresis, the DGGE gels were stained in 1X TAE solution containing ethidium bromide (50 μg ml− 1) for 15 min and photographed under a UV illumination using a Transilluminator Gel DocTM XR+ (BioRad, California, U.S.A.). The composition of PCR mix is given in the **Table 5**. and the recipe for DGGE gel is shown in the **Table 6.**

Obtained electropherograms were interpreted by cluster analysis, which seeks for similarity patterns among tested samples and produced dendrogram. The pictures of DGGE gels (1, 2 and 3) is provided in the Figure 9, 10 and 11.

There were also cut DNA from bands of choice from the gel and they were send for identifying presented bacteria by Sanger sequencing. The data acquired from Sanger sequencing were processed in Geneious software (Version 11.0.5; Biomatters Limited) and sequences were compared with Standard Nucleotide BLAST (**National Center for Biotechnology Information** 2018). The obtained DGGE microbial profiles from faecal and rumen samples were processed in BioNumerics software (version 9.1; Applied Maths NV, Sint-Martens-Latem, Belgium). The processing included compensation of differences among the intensity of the lanes (normalisation) and calculation of the correlation matrix. Clustering was done with Pearson correlation and the UPGMA method [Principal Coordinates Analysis (PCoA)].

Table 5: Composition of PCR mix.

DNA template	1 uL
Forward primer 10x338GC	1 uL
Reverse primer 10x534RP	1 uL
PCR mix	15 uL
dH_2O	12 uL
Total	30 uL

Table 6: Recipe for DGGE gel.

DGGE		**25 ml**
Rate of denaturation	**35%**	**60%**
40% Akrylamide (ml)	5.560	5.560
50x TAE (ml)	0.500	0.500
Formamide (ml)	3.500	6.000
Urea (g)	3.675	6.300
H_2O (ml)	12.250	9.500

3.3.1.6. NGS

3.3.1.6.1 Amplification of 16S rRNA

For the amplification of 16S rRNA there was used 20 ng of DNA for preparation of PCR amplicons of V4-V5 region according to **Fliegerova et al**. (2014). To the mixture there was added OneTaq DNA Polymerase (New England Biolabs). The thermal cycles of PCR were composed of initial denaturation for 5 minutes at 95 °C, followed by 35 cycles of 30 seconds at 95 °C, 30 seconds at 57 °C and 30 seconds at 30 seconds at 72 °C, last cycle was final elongation which last 5 minutes at 72 °C. Acquired PCR amplicons were tested at 1.5% agarose electrophorese for 25 minutes at 90 V. Then they were purified by "QIAquick® PCR Purification Kit" (Qiagen company) according to the protocol. The quality of samples (concentration of nucleic acids) was tested by microvolume spectrophotometer (NanoDrop OneC, Thermo Fisher Scientific Inc.).

3.3.1.6.2 Next Generation Sequencing

According to **Milani et al.** (2013) acquired PCR products were used for the preparation of amplicon libraries for diversity analyses by next-generation sequencing approach on Personal Genome Machine (Life Technologies). For the preparation of sequencing libraries, there were used 200 ng DNA from each sample. Procedure was done with the use of KAPA Library Preparation kit (Kapa Biosystems) according to manufacturer's protocol. Each sample was labeled by the Ion Xpress Barcode adapters (Thermo Fisher Scientific). For the quantification of acquired libraries we used KAPA Library Quantification Kit (KAPA BIOSYSTEMS). Quantified libraries were used for preparation of sequencing template with the use of Ion PGM™ Hi-Q™ View OT2 Kit (Thermo Fisher Scientific) on the Ion OneTouch™ 2 instrument. Then we used Ion 316™ Chip Kit v2 (Thermo Fisher Scientific) for sequencing of template using Ion PGM™ Hi-Q™ View Sequencing kit (Thermo Fisher Scientific).

3.3.1.6.3 Data analyses of NGS

We obtained sequences in the FASTQ format and they were further processed by QIIME analyses pipeline (Caporaso et al. 2010). Samples shorter than 250 base pair and singletons were removed, sequences were also quality filtered. We also removed chimeras by USEARCH tool (**Edgar** 2010). The rest of the sequences were clustered and identified by performing open-reference OTU picking against the Greengene database (**DeSantis** et al. 2006). We used QIIME (open source software) for computing diverisity

index, unweighted and weighted UniFrac distance metrics analysis (**Lozupone** et al. 2005) and the results were illustrated by principle coordinate analysis (PCoA).

3.3.2. Used machines

In the Table 7 there is a description of instruments used during the analyses, including company name and for which procedure they were used.

Table 7: Instruments used for laboratory analysis.

Instrument	**Company**	**Procedure**
FastPrep®-24 Classic Instrument	MP Biomedicals	Vortexing
NanoDrop™ One C Microvolume Spectrophotometer	Thermo Fisher Scientific	DNA concentration
omniPAC Midi CS-300V power supply	Cleaver	Electrophoresis
MultiSUB Choice horizontal gel electrophoresis units	Cleaver	Electrophoresis
Transilluminator Gel DocTM XR+	BioRad	Gel Imaging
PowerPac™ Basic Power Supply	BioRad	DGGE
DCode™ Universal Mutation Detection System	BioRad	DGGE
Biometra TAdvanced	Analytik Jena AG	PCR
Hawk 15/05 Refrigerated Centrifuge	Sanyo, MSE	Centrifuge
Ion OneTouch™ 2	Life technologies	NGS
Ion OneTouch™ ES	Life technologies	NGS
Ion Torrent™ Ion Personal Genome Machine™ (PGM™) System	Life technologies	NGS

4. Results

DNA isolated from samples was successfully amplified by PCR which targeted the V3 regions (DGGE) and regionV4-V5(NGS) of the 16S rRNA. Produced amplicons were purified and the quality of obtained samples was evaluated on NanoDrop microvolume spectrophotometer and by gel electrophoresis, the gel picture is given in the Figure 8.

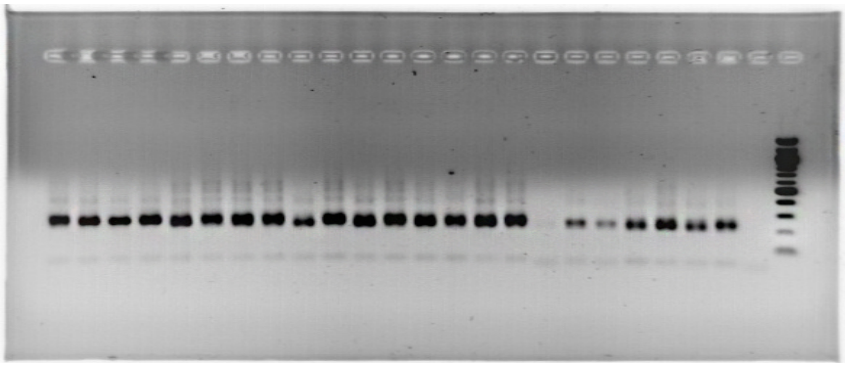

Figure 8: Picture of gel from electrophoresis.

4.1. DGGE

The acquired PCR products were used for DGGE analysis to detect whether the microbiome changes according to different diets and whether it differs between faecal and rumen samples. DGGE profiles of microbiome of sampled elands fed by three various diets are shown in the Figures 13 and 14.

Relationship of faecal and rumen DGGE microbial profiles is shown in the dendrogram created in the BioNumerics software (**Figure 9**). Identified bands were marked by black arrows and labels which are corresponding to the field “label” in Table 8. There is clearly visible the similarity of profiles from animals fed by control diet, as well as similarity of rumen microbial profiles. However, the differences of hay diet and alfalfa silage diet are not so clear, which could be caused by higher variations of microbiome between individuals than between diets.

Twenty amplicons were excised from the DGGE gels and sequenced. Obtained sequences were compared with Standard Nucleotide BLAST (**National Center for Biotechnology Information** 2018). We have chosen 5 representative bands which we were identified. The similarities to NCBI Reference Sequence, diet related to identified sequences and sample numbers are described in the Table 8. Genus *Acinetobacter* was

found in the sample K13 from anonymous eland on third (control) diet. In the sample K6 from the animal named Hanno, fed by control diet, there was present *Neptunitalea chrysea*, *Ercella succinigenes* and *Romboutsia lituseburensis*.

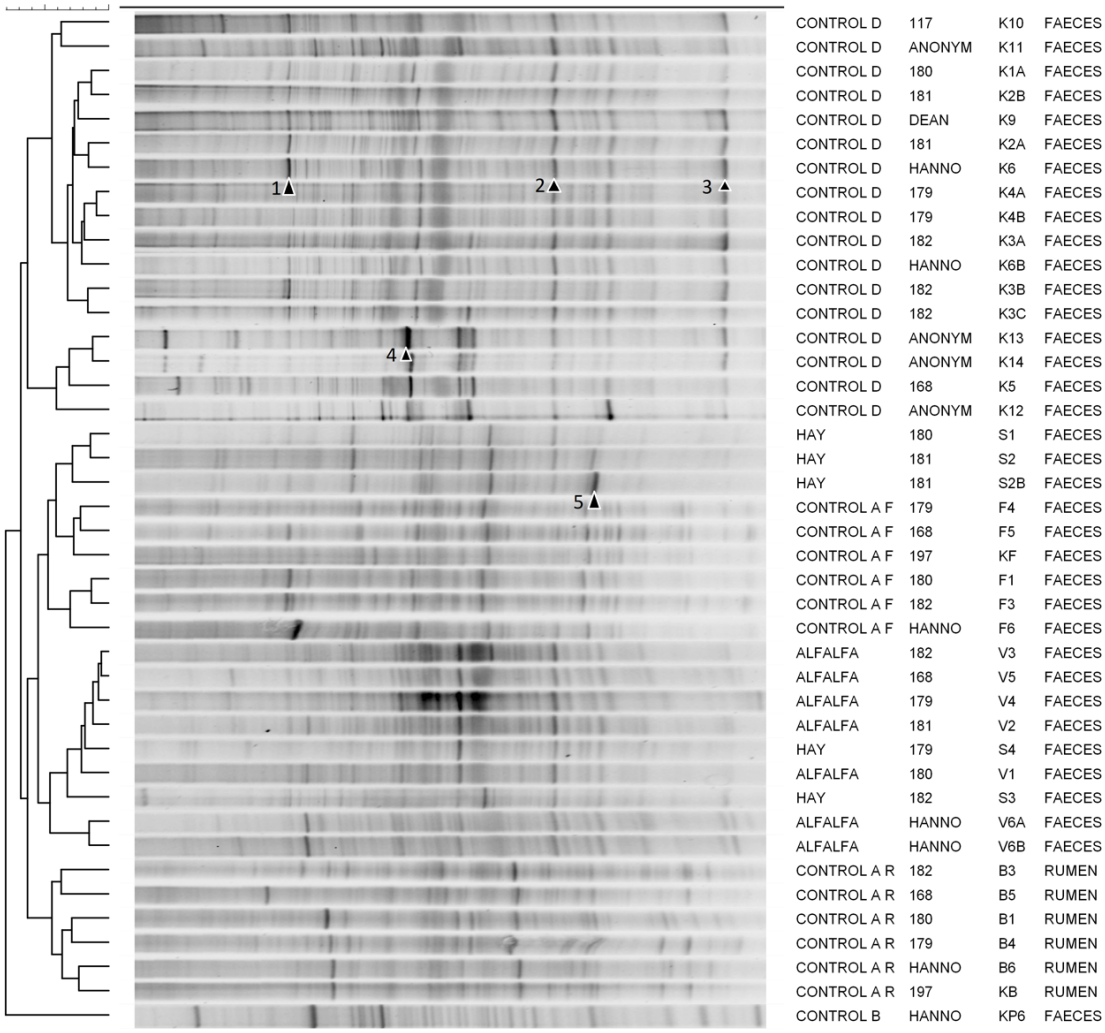

Figure 9: Dendrogram of faecal and rumen samples from DGGE profiles. Bands identified according to NCBI are marked by black arrows and numbered labels. Labels are corresponding to the labels in Table 8.

Table 8: Description of sequenced samples, identified by NCBI – Label of identified band, identification of microorganism, similarity and accession to NCBI Reference Sequence, diet and sample number.

Label	Identification NCBI	Similarity	Accesion	Diet	Sample
1	*Acinetobacter*	98 %	NR_113346.1	Control	K13
2	*Neptunitalea chrysea*	84 %	NR_145556.1	Control	K6
3	*Ercella succinigenes*	84 %	NR_134026.1	Control	K6
4	*Romboutsia lituseburensis*	95 %	NR_118728.2	Control	K6
5	*Stenotrophomonas koreensis*	99 %	NR_041019.1	Hay	S2B

Phylogenetic tree of identified sequences is further illustrating the relationship of identified bacteria (Figure 10).

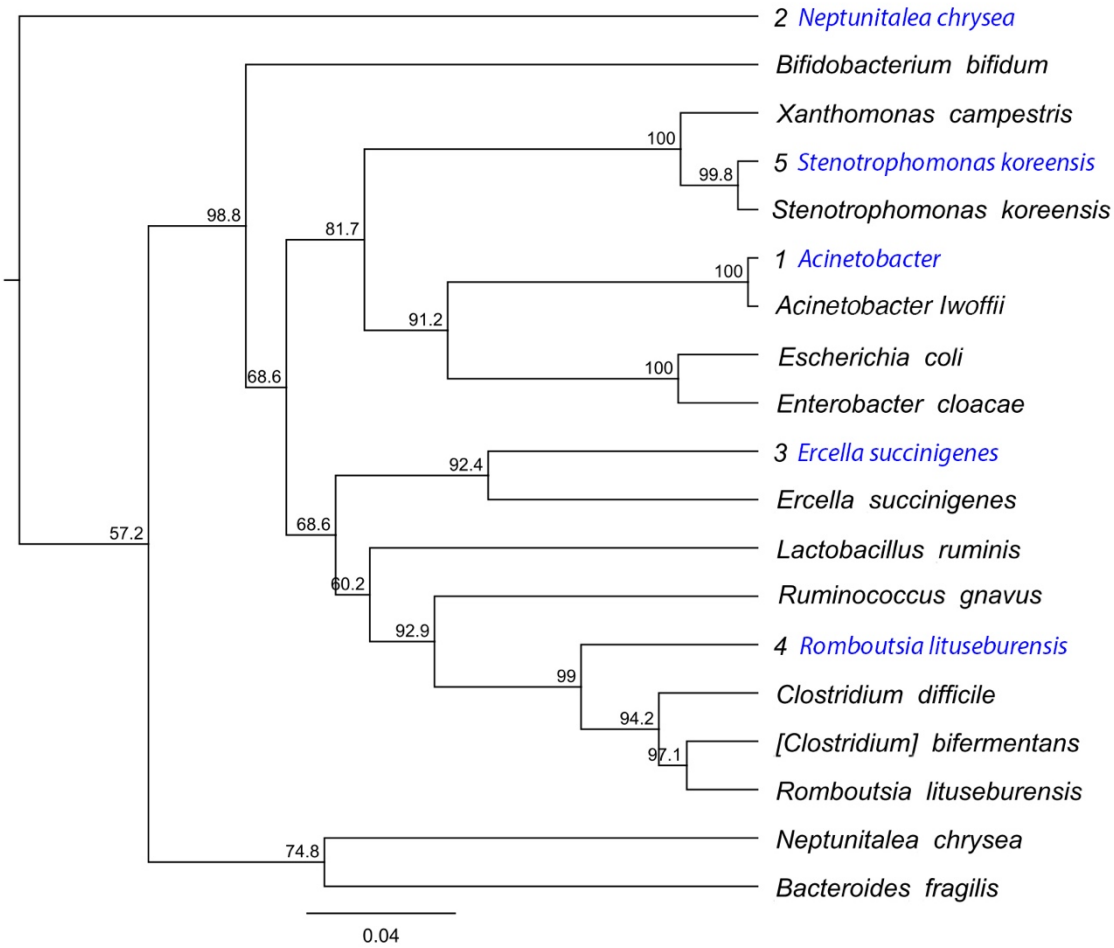

Figure 10: Phylogenetic tree of identified bands according to NCBI. The identified microorganisms are written in blue color. Numbered labels are corresponding to the field "label" in Table 8.

However, on the graph of the Principal Component Analysis (PCoA) the changes of microbiome due to varied diets are much clearer (Figure 11). The most distinct is the groups of yellow points representing control diet during the experiment. The group of green points, representing alfalfa diet, is also well defined. Just one blue point, representing hay diet, is included in the same patch as alfalfa hay, which could be, as noted before, due to variations of the microbiome of some individual animal.

The differences of rumen and faecal microbiome are also quite evident in the PCoA graph shown in Figure 12.

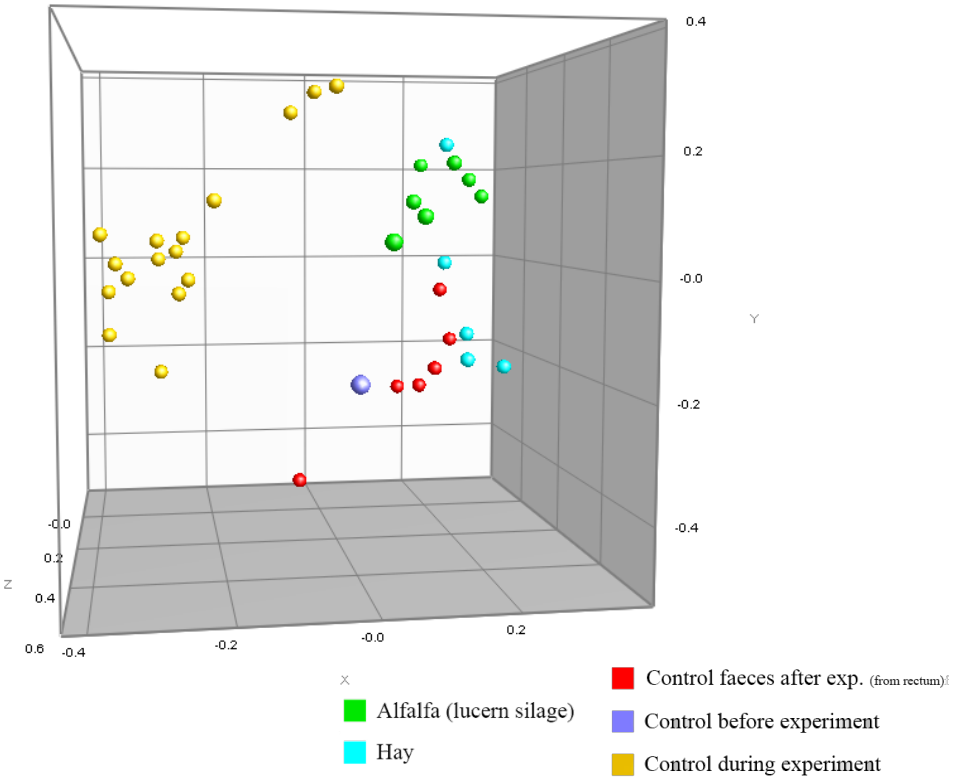

Figure 11: Principal Coordinates Analysis (PCoA) of all faecal samples according to different diets, Alfalfa, Hay and Control (before, during and after experiment).

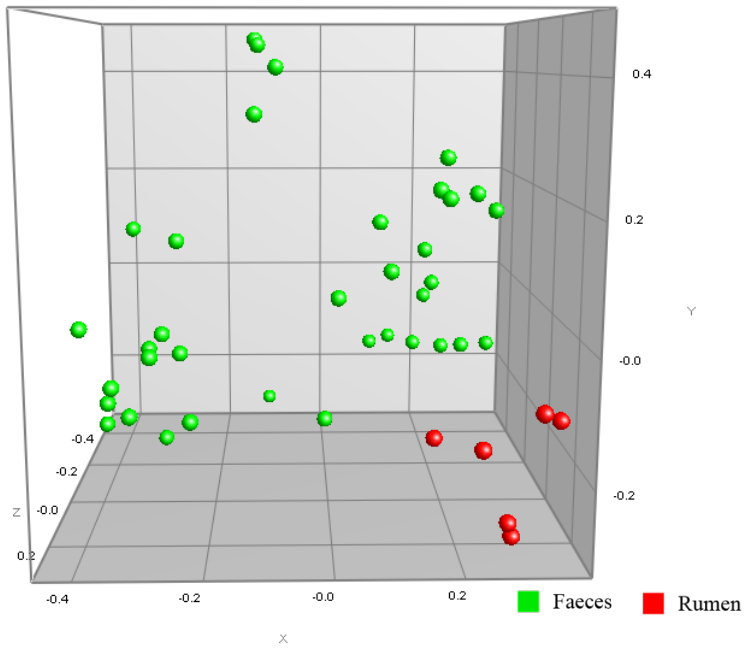

Figure 12: PCoA comparing all rumen and faecal samples.

4.2. NGS

The following taxonomical data were obtained by high throughput NGS analyses. The data were processed by the Qiime software. The vast majority of microorganisms in all categories (faecal microbiome of three various diets and rumen microbiome) were Bacteria (from 98.31% to 98.89%), Archaea comprised just 0.01-0.04% and the rest of the sequences were unassigned (Table 9; Figure 13).

Table 9: Abundance (%) representation of Archaea and Bacteria in all groups of samples

	Alfalfa silage	Grass hay	Control after exp.	Control rumen	Control during exp.
Unassigned	1.10%	1.14%	1.47%	1.66%	1.13%
Archaea	0.01%	0.01%	0.04%	0.03%	0.04%
Bacteria	98.89%	98.85%	98.50%	98.31%	98.84%

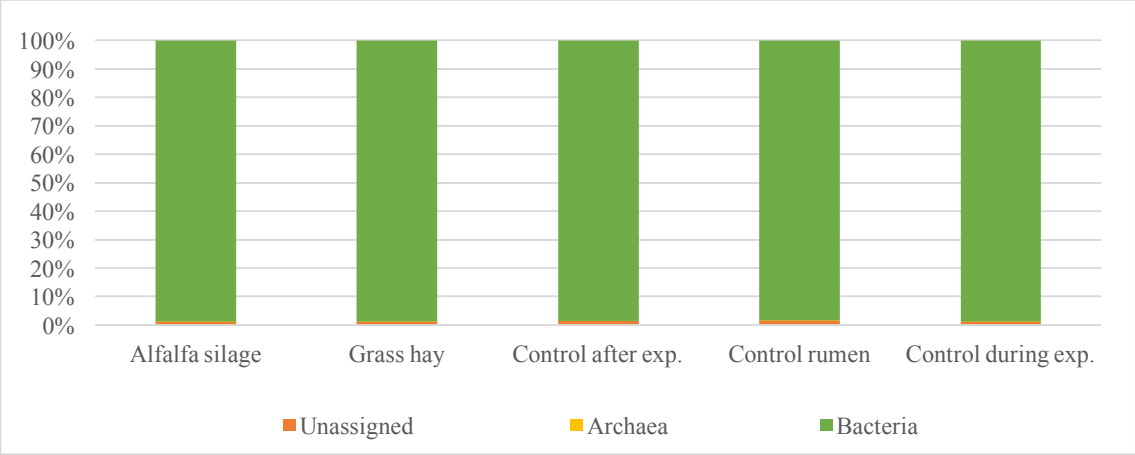

Figure 13: Abundance (%) of Archaea and Bacteria in all groups od samples.

4.2.1. The composition of microbiome on the phylum level

In all faecal samples, the most abundant phyla were Firmicutes (58-70%), on the other hand the dominant phylum in rumen samples were Bacteroidetes (60.22%).

In the faecal samples from diet composed of alfalfa silage the most abundant phyla were Firmicutes (70.18%), second most abundant were Bacteroidetes (25.11%). Other present phyla were Tenericutes (0.78%), Proteobacteria (0,65%) and Saccharibacteria - TM7 (0.58%).

In the faecal samples from diet composed of grass hay Firmicutes comprised 58.37%, Bacteroidetes 19.85%, however phylum Proteobacteria was also significantly more abundant then in all other diets (17.62%). Other present phyla were Tenericutes (0.47%) and Saccharibacteria - TM7 (0.40%).

The microbiome of samples from common daily diet (control during experiment) Was the most similar to the microbiome of alfalfa silage samples, with the main difference in the percentage of Proteobacteria (only 0.65% in alfalfa, but 2.57% in control d. exp.).

Microbiome of control samples collected after experiment from the rectum was little bit shifted from the control collected during experiment (Table 10). Rumen microbiome differs from faecal mainly because of the shift in dominant phylum – in the rumen dominant phylum are Bacteroidetes (60.22%) and Firmicutes form only 32.34%, but in faecal microbiome after experiment Firmicutes form 62.23% and Bacteroidetes only 32.57%.

Table 10: Differences in abundance (%) of microbiome on the phylum level.

Phylum	**Alfalfa silage**	**Grass hay**	**Control after exp.**	**Control during exp.**	**Rumen after exp.**
Unassigned	1.10%	1.14%	1.47%	1.13%	1.66%
Bacteroidetes	25.11%	19.85%	32.57%	20.17%	60.22%
Firmicutes	70.18%	58.37%	62.23%	70.96%	32.34%
Proteobacteria	0.65%	17.62%	0.48%	2.57%	0.73%
Saccharibacteria (TM7)	0.58%	0.40%	0.55%	0.66%	1.42%
Tenericutes	0.78%	0.47%	0.91%	0.83%	1.12%

4.2.2. The composition of microbiome on the class level

4.2.2.1. Grass Hay

The first most abundant class in grass hay diet microbiome were *Clostridia* (34.88%), second *Bacilli* (23.43%), third *Bacteroidia* (15.58%), forth *Gammaproteobacteria* (15.39%), fifth *Sphingobacteriia* (3.03%), sixth *Flavobacteriia*

(1.22%), seventh *Betaproteobacteria* (1.12%) and eighth *Actinobacteria* (1.03%). The abundance of all other classes was below 1% (Table 11).

4.2.2.2. Alfalfa silage

In the alfalfa diet (faecal) samples there were only three classes abundant over 1%. The most abundant were *Clostridia* (68.20%), then *Bacteroidia* (25.08%) and *Bacilli* (1.88%). All other present genera are described in the Table 11.

4.2.2.3. Rumen

In the rumen samples the most abundant classes were *Bacteroidia* (60.22%), *Clostridia* (32.0%), *TM7-3* (1.42%) and *Mollicutes* (1.11%).

4.2.2.4. Control

In the control during experiment the most abundant classes were *Clostridia* (66.01%), *Bacteroidia* (20.06%), *Bacilli* (4.87%), *Gammaproteobacteria* (2.06%) and *Actinobacteria* (1.79%). The control after experiment was less variable, the first most abundant class was Clostridia (61.75%) and the second most abundant was class *Bacteroidia* (32.57%), all other classes were abundant below 1%.

Class	Alfalfa silage	Control after exp.	Control rumen	Control during exp.	Grass hay
Unassigned	**1.10%**	**1.47%**	**1.66%**	**1.13%**	**1.14%**
Actinobacteria	0.04%	0.09%	0.27%	**1.79%**	**1.03%**
Bacteroidia	**25.08%**	**32.57%**	**60.22%**	**20.06%**	**15.58%**
Flavobacteriia	0.01%	0.00%	0.00%	0.09%	**1.22%**
Sphingobacteriia	0.01%	0.00%	0.00%	0.02%	**3.03%**
Fibrobacteria	0.27%	0.21%	0.54%	0.31%	0.09%
Bacilli	**1.88%**	0.34%	0.03%	**4.87%**	**23.43%**
Clostridia	**68.20%**	**61.75%**	**32.00%**	**66.01%**	**34.88%**
Alphaproteobacteria	0.27%	0.20%	0.10%	0.27%	0.91%
Betaproteobacteria	0.10%	0.03%	0.03%	0.05%	**1.12%**
Gammaproteobacteria	0.08%	0.08%	0.25%	**2.06%**	**15.39%**
Spirochaetes	0.76%	0.94%	0.59%	0.97%	0.41%
TM7-3	0.58%	0.55%	**1.42%**	0.66%	0.40%
Mollicutes	0.60%	0.80%	**1.11%**	0.74%	0.23%

5. Discussion

Results shown that microbiome profiles from faecal samples of eland differs due to the various diets and also that there is a significant difference between rumen and faecal samples. Similar results were described by **Omoniyi et al**. (2014), **Ley et al. (2008), Thursby & Juge (2017)** and **Henderson et al. (2015).** However, diet and the type of sample (rumen, faecal) are not the only factors which could affect the microbiome composition. For example **Rudi et al. (2012)** found significant changes of the faecal microbiome due to natural yearly fluctuations, where gender, diet, weather, breed or age, was not connected to the microbial differences between individuals. Major microbiome differences between individual animals, bred under equal conditions, are also described by **Durso et al.** (2012) and **Henderson et al. (2015).**

The majority of microorganisms in all categories of this research (faecal microbiome of three various diets and rumen microbiome) were composed from Bacteria (from 98.31% to 98.89% abundance) and Archaea comprised just 0.01-0.04%.

In all faecal samples in current study, the most abundant phyla were Firmicutes (58-70%). Similarly, **Tanca et al.** (2017) reported that faecal microbiome of sheep (*Ovis aries*) was formed over 80% by Firmicutes and Bacteroidetes. Also in the study of (**Rudi** et al. 2012) Firmicutes were dominant group of the microbiome of cattle (81.9% of all the reads), followed by Proteobacteria (9.6%), Bacteroidetes (5.4%), and Actinobacteria (2.8%).

The majority of all research was focused on microbial communities inhabiting rumen of cattle. Rumen microbiome is certainly influencing production and overall digestion more than faecal microbiome, but obtaining of rumen samples is always invasive procedure and requires either cannulation or slaughtering of animal. These techniques are not suitable for all species of animals (elusive or dangerous species, endangered animals etc.), because of this the evaluation of difference between faecal and rumen microbiome is also valuable.

The first most abundant class in grass hay diet microbiome were *Clostridia* (34.88%), second *Bacilli* (23.43%), third *Bacteroidia* (15.58%), forth *Gammaproteobacteria* (15.39%), fifth *Sphingobacteriia* (3.03%), sixth *Flavobacteriia* (1.22%), seventh *Betaproteobacteria* (1.12%) and eighth *Actinobacteria* (1.03%).

In the current study genus *Acinetobacter* (Proteobacteria) was found in the sample K13 from anonymous eland on third (control) diet. In the sample K6 from the animal named Hanno, fed by control diet, there was present *Neptunitalea chrysea* (**Bacteroidetes)**, *Ercella succinigenes* (sp. nov.) and *Romboutsia lituseburensis* (Firmicutes).

In this study, dominant phylum in rumen samples of eland were Bacteroidetes (60.22%) and the most abundant classes were Bacteroidia (60.22%), Clostridia (32.0%), TM7-3 (1.42%) and Mollicutes (1.11%).

Accornding to **Henderson et al. (2015)** the seven most dominant bacteria species inhabiting rumen were Prevotella, Butyrivibrio, and Ruminococcus, as well as unclassified Lachnospiraceae, Ruminococcaceae, Bacteroidales, and Clostridiales (67.1% of all bacterial sequence data). In the study of **Omoniyi** et al. (2014) microbial groups were highly diverse and dominated by the phyla Firmicutes, Bacteroidetes, Tenericutes, Actinobacteria and Proteobacteria.

Rumen communities in cattle were highly diverse and the most abundant phyla were Firmicutes, Bacteroidetes, Tenericutes, Actinobacteria and Proteobacteria (**Omoniyi** et al. 2014).

Bacteroidetes phylum was prevalent in all rumen samples based on OTUs distribution. Within Bacteroidetes phylum, Prevotellaceae family appeared most abundant in all samples **Patel et al**. (2014).

6. Conclusions

In conclusion, there were defined rumen and faecal microbiome of farmed common eland. There were major changes of microbiome due to varied diets as well as significant difference between rumen and faecal samples. In all faecal samples, the most abundant phyla were Firmicutes (58-70%), on the other hand the dominant phylum in rumen samples were Bacteroidetes (60.22%). Interesting difference between grass hay diet and alfalfa silage was in the phylum Proteobacteria, which were in the alfalfa diet represented only by 0.65%, but in the grass hay diet, their abundance was 17.62%. From the DGGE We have chosen 5 representative bands which we were identified. Genus *Acinetobacter* was found in the sample K13 from anonymous eland on third (control) diet. In the sample K6 from the animal named Hanno, fed by control diet, there was present *Neptunitalea chrysea*, *Ercella succinigenes* and *Romboutsia lituseburensis*.

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8. Appendices

List of the Appendices:

- **Appendix 1:** PowerFecal® DNA Isolation Kit Instruction Manual
- **Appendix 2:** QIAquick® PCR Purification Kit Protocol

Appendix 1: PowerFecal® DNA Isolation Kit Instruction Manual

PowerFecal®

DNA Isolation Kit

Catalog No.	Quantity
12830-50	50 Preps

Instruction Manual

Inhibitor Removal Technology® (IRT) is a registered trademark of MO BIO Laboratories, Inc. and is covered by the following patents USA US 7,459,548 B2, Australia 2005323451 and India 246946.

Please recycle

Technical Information: Toll free 1-800-606-6246, or 1-760-929-9911 Email: technical@mobio.com **Website: www.mobio.com**

Introduction

The PowerFecal® DNA Isolation Kit is designed for fast and easy purification of both microbial and host genomic DNA from stool and feces. Based on the MO BIO PowerSoil® DNA Isolation Kit, the PowerFecal® DNA Isolation Kit uses the same patented Inhibitor Removal Technology® (IRT) for stool that has worked so well for soil. IRT is very effective at removing inhibitory substances commonly found in stool such as polysaccharides, heme compounds and bile salts. The result is high purity DNA that is ready to use in the most demanding downstream applications.

Protocol Overview

The recommended starting sample is 0.25 grams of stool or biosolid. Each sample is homogenized in a 2 ml bead beating tube containing garnet beads. Cell lysis of host cells as well as microbial cells is facilitated by both mechanical collisions between beads and chemical disruption of cell membranes, ensuring efficient extraction from even the toughest of microorganisms. Patented Inhibitor Removal Technology® (IRT) is then used to remove common substances in fecal samples that interfere with PCR. Total genomic DNA is captured on a silica spin column. DNA is then washed and eluted so that it is ready for PCR analysis and other downstream applications including qPCR and next generation sequencing analysis.

High Throughput Options

MO BIO offers a vacuum based protocol for faster processing without centrifugation for the DNA binding and column washing steps for Spin Filters. The MO BIO PowerVac™ Manifold (Catalog# 11991) allows for processing of up to 20 spin filter preps at a time using the PowerVac™ Mini Spin Filter Adapters (See Other Related Products listed below). Using the PowerVac™ Manifold minimizes the most time consuming steps in the procedure. For additional high throughput options MO BIO offers the PowerSoil®-htp 96 Well Soil DNA Isolation Kit for processing 2 x 96 samples using a centrifuge capable of spinning two 96 Well Blocks stacked (13 cm x 8 cm x 5.5 cm) at 2500 x *g*. For 96 well plate homogenization, we recommend the Retsch 96 Well Plate Shaker (MO BIO

Catalog# 11996 in the USA only) and Adapters (MO BIO Catalog# 11990). For information outside the USA, contact technical@mobio.com.

This kit is for research purposes only. Not for diagnostic use.

MO BIO Laboratories, Inc.

PowerFecal® DNA Isolation Kit

Prepare Sample

- Add sample to Dry Bead Tube
- Add Bead Solution
- Add Solution C1
- Heat Tubes at 65°C
- Attach to Vortex Adapter
- Vortex

Centrifuge

Cell Lysis

- Add Solution C2
- Incubate at 4°C

Centrifuge

Inhibitor Removal Technology®

- Add Solution C3
- Incubate at 4°C

Centrifuge

Bind DNA

- Add Solution C4
- Load into Spin Filter

Centrifuge

Wash

- Wash with Solution C5

Centrifuge

Elute

- Elute with Solution C6

Other Related Products	Catalog No.	Quantity
PowerMax® Soil DNA Isolation Kit	12988-10	10 preps
PowerSoil®-htp 96 Well Soil DNA Isolation Kit	12955-4 12955-12	4 x 96 preps 12 x 96 preps
Ceramic Bead Tubes, 1.4 mm Glass Bead Tubes, 0.5 mm Glass Bead Tubes, 0.1mm	13113-50 13116-50 13118-50	50 tubes 50 tubes 50 tubes
PowerVac™ Manifold	11991	1 manifold
PowerVac™ Mini System	11992	1 unit + 20 adapters
PowerVac™ Mini Spin Filter Adapters	11992-10 11992-20	10 adapters 20 adapters
PowerLyzer® 2 ml Tube Holder	13156	1 unit
PowerLyzer® Tube Holder Stand	13157	1 unit

Equipment Required

Microcentrifuge (13,000 x *g*)

Pipettors (60 µl - 750 µl)

Vortex-Genie® 2 Vortex (MO BIO Catalog# 13111-V or 13111-V-220)

Vortex Adapter (MO BIO Catalog# 13000-V1-24)

Reagents Required but not Included

100% ethanol (for the PowerVac™ Manifold protocol only)

Kit Contents

	Kit Catalog # 12830-50	
Component	**Catalog #**	**Amount**
Dry Bead Tubes	12830-50-BT	50
Bead Solution	12830-50-BS	42 ml
Solution C1	12830-50-1	3.3 ml
Solution C2	12830-50-2	14 ml
Solution C3	12830-50-3	11 ml
Solution C4	12830-50-4	72 ml
Solution C5	12830-50-5	30 ml
Solution C6	12830-50-6	6 ml
Spin Filters (units in 2 ml tubes)	12830-50-SF	50
2 ml Collection Tubes	12830-50-T	200

Kit Storage

Kit reagents and components should be stored at room temperature (15-30°C).

Precautions

Please wear gloves when using this product. Avoid all skin contact with kit reagents. In case of

contact, wash thoroughly with water. Do not ingest. See Material Safety Data Sheets for emergency procedures in case of accidental ingestion or contact. All MSDS information is available upon request (760-929-9911) or at www.mobio.com on the product page. Reagents labeled flammable should be kept away from open flames and sparks.

WARNING: Solution C5 contains ethanol. It is flammable. Do not use bleach to clean the inside of the PowerVac™ Manifold or to rinse the PowerVac™ Mini Spin Filter Adapters when attached to the manifold.

IMPORTANT NOTE FOR USE: Shake to mix Solution C4 before use.

Experienced User Protocol

Please wear gloves at all times

1. To the Dry Bead Tube, provided, add 0.25 grams of stool or biosolid.
Note: For fecal samples that are especially high in lipids, polysaccharides and protein (for example: meconium or some bird feces) less material (0.10 grams) may improve the DNA yield and purity.

2. Add **750 µl of Bead Solution** to the **Dry Bead Tube**. Gently vortex to mix.

3. **Check Solution C1.** If **Solution C1** has precipitated, heat solution to 60°C until dissolved before use.

4. Add **60 µl of Solution C1** and invert several times or vortex briefly.

5. Heat the tubes at 65°C for 10 minutes.

6. Secure the bead tubes horizontally using the MO BIO Vortex Adapter tube holder for the vortex or secure tubes horizontally on a flat-bed vortex pad with tape. Vortex at maximum speed for 10 minutes.

7. Centrifuge the tubes at 13,000 x *g* for 1 minute.

8. Transfer the supernatant to a clean **2 ml Collection Tube** (provided). Expect between 400 to 500 µl of supernatant.

9. Add **250 µl of Solution C2** and vortex briefly to mix. Incubate at 4°C for 5 minutes.

10. Centrifuge the tubes at 13,000 x *g* for 1 minute.

11. Avoiding the pellet, transfer up to 600 µl of supernatant to a clean **2 ml Collection Tube** (provided).

12. Add **200 µl of Solution C3** and vortex briefly. Incubate at 4°C for 5 minutes.

13. Centrifuge the tubes at 13,000 x *g* for 1 minute.

14. Avoiding the pellet, transfer the supernatant to a clean **2 ml Collection Tube** (provided). Do not transfer more than 750 µl at this step.

15. Shake to mix Solution C4 before use. Add 1200 µl of **Solution C4** to the supernatant and vortex for 5 seconds.

16. Load 650 µl of supernatant onto a **Spin Filter** and centrifuge at 13,000 x *g* for 1 minute. Discard the flow through and repeat until all the supernatant has been loaded onto the Spin Filter.

    Note: A total of three loads for each sample processed are required.

    High Throughput Option: Step 16 can become tedious when many samples need to be processed. For this reason, MO BIO has developed a vacuum protocol. It does require the purchase of our aluminum Spin Filter Adapters (MO BIO Catalog# 11992-10) which will allow you to fit our flat bottom spin filters on to any vacuum manifold with Luer lock fittings. Please read **Vacuum Protocol using the PowerVac™ Manifold** on page 11.

17. Add 500 µl of **Solution C5** and centrifuge for 1 minute at 13,000 x *g*.

18. Discard the flow through.

19. Centrifuge again for 1 minute at 13,000 x *g*.

20. Carefully place the Spin Filter in a clean **2 ml Collection Tube** (provided). Avoid splashing any of **Solution C5** onto the **Spin Filter**.

21. Add 100 µl of **Solution C6** to the center of the white filter membrane. Alternatively, sterile DNA-Free PCR Grade Water or TE buffer may be used for elution from the silica Spin Filter membrane at this step (MO BIO Catalog# 17000-10).

Note: Eluting with 100 µl of Solution C6 will maximize DNA yield. For more concentrated DNA, a minimum of 50 µl of Solution C6 can be used. Do not use less than 50 µl of Solution C6.

22. Centrifuge at 13,000 x *g* for 1 minute and discard the Spin Filter basket.

The DNA in the tube is now ready for any downstream application. No further steps are required.

We recommend storing DNA frozen (-20° to -80°C). **Solution C6** contains no EDTA.

To concentrate the DNA see the Hints & Troubleshooting Guide.

Thank you for choosing the PowerFecal® DNA Isolation Kit.

Contact Information

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Phone MO BIO Laboratories, Inc. Toll Free 800-606-6246, or
760-929-9911 Email: technical@mobio.com
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Email: orders@mobio.com
Fax: 760-929-0109
Mail: MO BIO Laboratories, Inc, 2746 Loker Ave West, Carlsbad, CA 92010
For the distributor nearest you, visit our web site at www.mobio.com/distributors

Appendix 2: QIAquick® PCR Purification Kit Protocol

QIAquick® PCR Purification Kit

The QIAquick PCR Purification Kit (cat. nos. 28104 and 28106) can be stored at room temperature (15–25°C) for up to 12 months. For more information, please refer to the *QIAquick Spin Handbook*, *March 2008*, which can be found at: www.qiagen.com/handbooks. For technical assistance, please call toll-free 00800-22-44-6000, or find regional phone numbers at www.qiagen.com/contact.

Notes before starting:

- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- All centrifugation steps are carried out at 17,900 x *g* (13,000 rpm) in a conventional table-top microcentrifuge at room temperature.
- Add 1:250 volume pH indicator I to Buffer PB. The yellow color of Buffer PB with pH indicator I indicates a pH of ≤7.5. If the purified PCR product is to be used in sensitive microarray applications, it may be beneficial to use Buffer PB without the addition of pH indicator I. Do not add pH indicator I to buffer aliquots.

1. Add 5 volumes Buffer PB to 1 volume of the PCR reaction and mix. If the color of the mixture is orange or violet, add 10 µl 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn yellow.
2. Place a QIAquick column in S a provided 2 ml collection tube or into a vacuum manifold. For details on how to set up a vacuum manifold, refer to the *QIAquick Spin Handbook*.
3. To bind DNA, apply the sample to the QIAquick column and S centrifuge for 30–60 s or z apply vacuum to the manifold until all the samples have passed through the column. S Discard flow-through and place the QIAquick column back in the same tube.
4. To wash, add 0.75 ml Buffer PE to the QIAquick column S centrifuge for 30–60 s or z apply vacuum. S Discard flow-through and place the QIAquick column back in the same tube.
5. Centrifuge the QIAquick column once more in the provided 2 ml collection tube for 1 min to remove residual wash buffer.
6. Place each QIAquick column in a clean 1.5 ml microcentrifuge tube.
7. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0– 8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. For increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.

8. If the purified DNA is to be analysed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual.