Predominant fecal microbiota of captive adult cheetahs housed in European zoos: a cross-sectional study

Niloufar MAJDZADEH

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Promoter: Prof. Dr. Geert Huys
Mentor: drs. Anne Becker
Department of Biochemistry and Microbiology (WE10)
Laboratory of Microbiology
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Table of Contents

TABLE OF CONTENTS

Acknowledgments ........................................................................................................... 2
Abbreviation list ............................................................................................................... 6
Chapter I. Summary ........................................................................................................ 7
Chapter II. Introduction .................................................................................................. 8
  II.1. Gut microbiota ...................................................................................................... 8
    II.1.1. The intestinal tract as microbial ecosystem .................................................... 8
    II.1.2. Function of gut microbiota ............................................................................. 9
    II.1.3. Role of diet on gut microbiota ..................................................................... 12
  II.2. Techniques for studying diversity of gut microbiota ............................................. 14
  II.3. Cheetah (Acinonyx jubatus) ................................................................................ 17
    II.3.1. Classes based on dietary habits .................................................................... 17
    II.3.2. Vulnerable status of cheetahs ...................................................................... 18
    II.3.3. Cheetah’s diet and its role in cheetah’s metabolism ....................................... 19
    II.3.4. Domestic cat as a model for studying cheetah gut microbiota ...................... 20
Chapter III. Goals .......................................................................................................... 23
Chapter IV. Results ......................................................................................................... 24
  IV.1. DGGE data .......................................................................................................... 24
    IV.1.1. Analysis of all DGGE fingerprints ................................................................. 26
    IV.1.2. Band richness of the DGGE fingerprints per zoo ........................................... 32
  IV.2. Linking the DGGE data with the clone library .................................................... 35
    IV.2.1. Common band-classes in the fingerprints ...................................................... 35
    IV.2.2. Linking band-classes with fingerprint clusters .............................................. 36
  IV.3. quantitative-PCR results ...................................................................................... 40
    VI.3.1. The quantity of Clostridium cluster XIVa per zoo ......................................... 40

3
Table of Contents

VI.3.2. The quantity of *Clostridium* cluster XIVa per animal ............................................. 41

VI.3.3. *Clostridium* cluster XIVa versus the DGGE band intensity ....................................... 41

Chapter V. Discussion .............................................................................................................. 43

Chapter VI. Conclusion ........................................................................................................... 46

Chapter VII. Materials and methods ...................................................................................... 47

 VII.1. Fecal sample collection ................................................................................................ 47

 VII.2. Homogenization of fecal samples .................................................................................. 47

 VII.3. DNA extraction ............................................................................................................. 47

 VII.4. DNA quantity and quality check .................................................................................. 47

 VII.4.1. Agarose gel electrophoresis ..................................................................................... 47

 VII.4.2. Optical density measurement by spectrophotometer .................................................. 48

 VII.5. V3-16s rRNA PCR ....................................................................................................... 48

 VII.6. DGGE analysis and gel processing ............................................................................... 49

 VII.7. Data analysis ............................................................................................................... 49

 VII.8. quantitative PCR ......................................................................................................... 50

 VII.8.1. Calibration of a standard curve ................................................................................ 50

 VII.8.2. Quantifying *Clostridium* cluster XIVa in the fecal samples .................................. 51

Chapter VIII. References ....................................................................................................... 52

Chapter IX. Addendum ............................................................................................................ 63

 IX.1. Fecal samples collection ............................................................................................... 64

 IX.2. Homogenization of the fecal samples .......................................................................... 64

 IX.3. DNA extraction ............................................................................................................. 64

 IX.4. DNA quantity and quality check .................................................................................. 65

 IX.4.1. Agarose gel electrophoresis ..................................................................................... 65

 IX.4.2. Optical density measurement by spectrophotometer ............................................... 65
Table of Contents

IX.5. V3-16s rRNA PCR.................................................................65
IX.6. DGGE .................................................................................66
IX.7. Band extraction and sequencing ......................................................67
IX.8. q-PCR ....................................................................................67
   IX.8.1. Building a standard curve .........................................................67
   IX.8.2. Quantification of Clostridium cluster XIVa ............................68
# Abbreviations

## Abbreviation List

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFU</td>
<td>Colony-Forming Unit</td>
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<tr>
<td>MAMP</td>
<td>Microbe-Associated Molecular Pattern</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-Like Receptor</td>
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<tr>
<td>SCFA</td>
<td>Short-Chain Fatty Acid</td>
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<tr>
<td>EAZA</td>
<td>European Association of Zoos and Aquaria</td>
</tr>
<tr>
<td>DGGE</td>
<td>Denaturing Gradient Gel Electrophoresis</td>
</tr>
<tr>
<td>GALT</td>
<td>Gut-Associated Lymphoid Tissue</td>
</tr>
<tr>
<td>TH17</td>
<td>T Helper 17 cell</td>
</tr>
<tr>
<td>TTGE</td>
<td>Temporal Temperature Gradient Gel Electrophoresis</td>
</tr>
<tr>
<td>TGGE</td>
<td>Temperature Gradient Gel Electrophoresis</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction Fragment Length Polymorphism</td>
</tr>
<tr>
<td>ARISA</td>
<td>Automated Ribosomal Intergenic Spacer Analysis</td>
</tr>
<tr>
<td>SSCP</td>
<td>Single-Strand Conformation Polymorphism</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative PCR</td>
</tr>
<tr>
<td>OTU</td>
<td>Operational Taxonomic Units</td>
</tr>
<tr>
<td>PL</td>
<td>Planckendael</td>
</tr>
<tr>
<td>GA</td>
<td>Gaia Zoo</td>
</tr>
<tr>
<td>OV</td>
<td>Zoo Parc Overloon</td>
</tr>
<tr>
<td>BB</td>
<td>Safaripark Beekse Bergen</td>
</tr>
<tr>
<td>EB</td>
<td>Ree Park Ebeltoft Safari</td>
</tr>
<tr>
<td>SI</td>
<td>Réserve Africaine de Sigean</td>
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The gastrointestinal tract of mammals is associated with a diverse, dynamic and complex microbial community, in which the biggest group is represented by bacteria. The gut microbiota is responsible for production of essential vitamins, differentiation of the immune system, tissue homeostasis, energy harvest and short-chain fatty acid production. The cheetah (Acinonyx jubatus) is an endangered animal with limited metabolic ability to digest anything except proteins. This carnivore hunts gazelle, rabbit or antelope in the wild. However, they are mostly fed either meat or prey in captivity. One of the biggest problems that endanger cheetahs is the feeding mismanagement in the zoos that increases suboptimal health, gastrointestinal and metabolic diseases. Diet, microbiota and health are strongly correlated and several studies have been conducted on the diet and health of these carnivores. Yet, there is not sufficient information about their gut microbiota, which form the main go-between of diet and health. Since the current information about the gut microbiota of captive cheetahs has been obtained from 2 animals in Belgium, we aim to collect more fecal samples from animals in different zoos with various conditions. The aim is to investigate the diversity of their gut microbiota and whether this analysis reveals the presence of a core microbiota.

Using population fingerprinting by denaturing gradient gel electrophoresis (DGGE) of V3-16S rRNA amplicons in a cross-sectional study, we studied the fecal microbial diversity of 55 fecal samples from 36 captive cheetahs in 6 members of EAZA zoos. After homogenization of the fecal samples, community DNA extraction was carried out followed by V3-16S rRNA PCR. Afterwards, DGGE profiling was performed followed by data analysis. The fingerprints were compared with the available clone library and phylogenetic tree, previously built based on two captive adult cheetahs in Belgium. Afterwards, Quantitative PCR (qPCR) was performed in order to quantify Clostridium cluster XIVa.

As a result of DGGE analysis, there was no zoo-specific or diet type specific clustering in the DGGE fingerprints, which suggested that the fingerprints are affected by a combination of factors such as zoo, diet, genetic background and housing. Clostridium cluster XIVa, XI and I were present in almost all the DGGE profiles, which indicate that these clusters might be members of the core microbiota of these 55 fecal samples. Band-classes 28.3% and 41.3% did not correlate with any clone in the clone library, which belonged to Lactobacillus spp. after sequencing. Furthermore, the boxplot analysis of the fingerprints revealed the variation of the predominant bacterial diversity between the fingerprints per zoo, per animal and per diet type. The quantification of Clostridium cluster XIVa depicted that all the DNA samples consisted of this cluster except for one animal that suffered from a renal disease, which may probably be one of the reasons that this cluster was not detectable.

In the future studies, more fecal samples could be collected and analyzed by DGGE to confirm the diversity and the gut microbiota in captive adult cheetahs. Moreover, more bands, which belong to potentially discriminating band-classes, can be sequenced to identify the unknown bacterial genera and species in the clone library. Since multiple taxa can occupy the same position in the DGGE gel, as it is one of the shortcomings of DGGE, more zoomed DGGE can be performed.
CHAPTER II. INTRODUCTION

II.1. GUT MICROBIOTA

II.1.1. THE INTESTINAL TRACT AS MICROBIAL ECOSYSTEM

The gastrointestinal tract of mammals, including humans, is an organ system that starts from the mouth and ends with the anal canal (Martin, 2010) (Figure 1). The intestine, one essential organ in this system in which the mucosal immune system operates, has several fundamental tasks in humans. One is nutrient absorption made possible by its 200m² total surface area in humans (Hooper et al, 2012). The other is to provide an environment in which bacteria can grow and survive, which is done through the few micrometers of mucus layer between the bacterial community and the layer underneath (O’Hara & Shanahan, 2006).

The gastrointestinal tract is not only associated with eukaryotic tissues and cells as in other organs, but also with a diverse, dynamic and complex microbial community, in which the biggest group is represented by bacteria. The gastrointestinal tract of human and mammals also includes archaea, viruses, fungi and protozoa (Hooper et al, 2002). These bacteria are spread in almost all parts of the gastrointestinal tract of most mammals, with a relatively low intensity in the proximal and middle small intestine and high intensity in the large intestine. The number of the bacteria reaches to $10^{11}$–$10^{12}$ CFU/g in the human colon (Mackie et al, 1999) (Figure 1).

The human gastrointestinal tract (Matamoros et al, 2013) is sterile at birth (Matamoros et al, 2013). The bacteria start colonization of the host gastrointestinal tract during birth from the mother, the surrounding environment, infant feeding, gestational age, hospitalization, and antibiotic use by the infant (Greiner & Bäckhed, 2011; Penders et al, 2006). Therefore, the microbial composition of gut microbiota changes after birth both in diversity and richness throughout development. The diversity and richness of the gut microbiota in each individual reaches its highest complexity and homeostatic climax composition in adulthood (Sommer & Bäckhed, 2013; Scholtens et al, 2012). The gut microbiota remain relatively stable during most of adult life and is expected to become less sensitive to modification (Jalanka-Tuovinen et al, 2011).

Both anaerobic and aerobic bacteria exist in the gastrointestinal tract of humans in which those that are anaerobic, such as Bacteroides and Clostridium (Vedantam & Hecht, 2003), are dominant by 100- to 1,000-fold over the aerobic and facultative anaerobic bacteria in the human gastrointestinal tract (Clemente et al, 2012). In total, the human intestinal microbiota consists of approximately 500–1,000 species that belong to only a few of the known bacterial phyla (Qin et al, 2010) (Figure 1). Amongst them, Bacteroidetes and Firmicutes account for more than 90% of all the phyla of human intestinal bacteria (Tehrani et al, 2012).
Introduction

Human gastrointestinal tract harbors eukaryotes, viruses, archaea and bacteria. Culture-based techniques have illustrated that all healthy human adults share most of the same gut bacterial species referred to as a core microbiota. For instance, Escherichia coli can be isolated from most people. However, culture-independent techniques have demonstrated a vast microbial diversity with high variability over time and across populations (Knight et al, 2012). Studies have shown that Bacteroidetes and Firmicutes are the phyla in human adults that dominate their microbiota, whereas Actinobacteria, Proteobacteria and Verrucomicrobia are generally minor phyla (Eckburg et al, 2005). Additionally, the diversity of gut microbiota is hugely influenced by age, genetics, environment, diet, human health and medicine (Knight et al, 2012).

II.1.2. FUNCTION OF GUT MICROBIOTA

Microbiota have coevolved over millions of years with mammalian hosts (Ley et al, 2008). A balanced gut microbiota are important for host health due to its important role in numerous developmental, nutritional, physiological and immunological processes (Mackie et al, 1999; Hooper et al, 2001). The most important functions are:

Differentiation of the immune system: The gut microbiota is essential for differentiation of the immune system in the host through providing signals. These signals promote the maturation of immune cells and the normal development of immune functions in order to eventually protect the host against invasion by opportunistic pathogens and to suppress inflammatory responses (Chow et al, 2010; Smith et al, 2007; Round et al, 2011). It has been shown that both the innate and adaptive immune systems in mammals have evolved to require microbial interactions through their development (Chow et al, 2010; O’Hara & Shanahan, 2006). The human innate immune system recognizes general microbe-associated molecular patterns (MAMPs) that are parts of the bacteria such as their flagella or their cell walls. These antigens are recognized by the host Toll-like receptors (TLRs). In case of
Introduction

absence or mutation of TLRs, the gut and mucosal immune systems do not develop normally in human (O’Hara & Shanahan, 2006). The human gut microbiota also suppress inflammatory responses and promote immunological tolerance through TLRs (O’Hara & Shanahan, 2006). The human gut microbiota also help educating the T cells in the adaptive immune system to discriminate the self from non-self-cells to protect the body from killing its own cells, which leads to autoimmunity diseases (Kuhn & Stappenbeck, 2013) (Figure 2).

Production of essential vitamins: Production of vitamins is one of the many functions of gut microbiota in mammals (Smith et al, 2007; LeBlanc et al, 2013). Yet, some vitamins are not synthesized by the host. Therefore, these vitamins should be obtained from external sources with the help of the gut microbiota (Hill, 1997). Biotin (B12) is one of the many water-soluble vitamins, in which our body absorbs through the help of gut microbiota. Vitamin B12 is another important water-soluble vitamin that is crucial for DNA synthesis and is also produced by the gut microbiota by consumption of proteins since B12 is bound to proteins. Vitamin K, a fat-soluble vitamin, is of high importance for blood clotting and if absent the host may die due to bleeding. The daily requirement of this vitamin comes from the gut microbiota in mammals (Hill, 1997). These vitamins are synthesized by several intestinal bacterial genera, including Bacteroides, Eubacterium, Propionibacterium, and Fusobacterium (Hooper et al, 2002; Wilson & Nicholson, 2009).

Tissue homeostasis: This function involves a tightly regulated cell cycle and requires a balance between cell renewal and cell death, which is mediated by the gut microbiota in mammals (Sommer & Bäckhed, 2013). Studies conducted on mice have shown that TLR signals derived from the gut microbiota are required for regaining tissue homeostasis following injury in the intestine (Rakoff-Nahoum et al, 2004). Moreover, gut microbiota can influence tissue homeostasis in bones by decreasing the bone mass when more Ca2+ is needed in the body through recruiting specific cells called “osteoclasts”. These cells that are present in bones, are responsible for resorption of boney tissues when the body needs calcium ion (Sommer & Bäckhed, 2013) (Figure 2). The gut microbiota modulate bone homeostasis through modulation of T cell function, serotonin levels and cytokine (Sjogren et al, 2012).

Energy harvest and short-chain fatty acid production: Carbohydrates are important energy sources for human and microbial cells, in which the human enzymes cannot degrade most complex carbohydrates and plant polysaccharides (Treemaroli & Bäckhed, 2012). Therefore, the undigested and unabsorbed dietary carbohydrates, such as fibers, reach the colon and are fermented by the microbiota, resulting in the production of CO2, H2 and most importantly “short-chain fatty acids” (SCFA), which are organic fatty acids with 1 to 6 carbon atoms. Acetate, propionate and butyrate are the main short-chain fatty acids with certain advantages for the human and mammal’s body (Willing & Van Kessel, 2007; Hijova & Chmelerova, 2007). The production rate of short-chain fatty acids in several mammal’s body, is the highest for butyrate, while is the lowest for acetate, with the ratio of 3 to 1 (Topping & Clifton, 2001). Short-chain fatty acids are rapidly absorbed in the cecum and colon and only 5-10% is eventually excreted in the feces (Macfarlane & Macfarlane, 2003). It has been shown that SCFAs are associated with reduced risk of inflammatory bowel disease,
Introduction

cardiovascular diseases and different sorts of cancers such as colon cancer (Floch & Hong-Curtiss, 2001; Radulian et al, 2009).

Human enterocytes and colonocytes use butyrate as the major substrate to maintain energy producing pathways and 70-90% of this metabolite is metabolized by the colonocytes (Hijova & Chmelarova, 2007). Butyrate is regarded as a metabolite that is beneficial for the host health due to its positive influences on cell growth, differentiation and anti-inflammatory effects (Hamer et al, 2008). Inhibition of the transcription factor NF-kB, which is implicated in innate immunity, cell cycle control and apoptosis, is amongst the anti-inflammatory effects of butyrate (Luhrs et al, 2001; Segain et al, 2000). Butyrate also regulates cell proliferation in mammals through the release of growth factors or gastrointestinal peptides or by modulation of mucosal blood flow. Moreover, butyrate is the main short-chain fatty acid that can act directly on genes that regulate cell proliferation (Blottiire et al, 2003).

Acetate and propionate are taken up by the portal circulation and have an impact on lipid metabolism in an opposite manner. While acetate contributes to lipid and cholesterol synthesis in liver, propionate inhibits the effects of acetate (Laparra & Sanz, 2010). In addition, liver cells metabolize residual butyrate and propionate in order to use them in glycogenesis. While 50-70% of acetate is taken up by the liver, residual acetate is used by the muscles in order to generate energy (Hijova & Chmelarova, 2007). Moreover, several studies have shown that acetate is considered to be the predominant short-chain fatty acid, in concentration in the human colon. Yet, butyrate has the most beneficial effects on human health (Tedelind et al, 2007).

Many factors determine the production of short-chain fatty acids in the gut such as the transit time (Hijova & Chmelarova, 2007), substrate source (Cook & Sellin, 1998) and bacterial composition in the colon (Roberfroid, 2005). The fermentation takes place in the proximal colon since the substrate availability is the greatest (Hijova & Chmelarova, 2007). By the increase in the amount of short-chain fatty acids in the human gut, several beneficial bacterial species, such as Lactobacillus and Bifidobacterium, which are associated with improved health, increase as well (Roy et al, 2006). In contrast to most clostridial species in the mammal’s gastrointestinal tract, Lactobacillus and Bifidobacterium are generally regarded as beneficial microbes due to their ability to eliminate harmful bacteria and pathogens by producing various antimicrobial agents such as antibiotics (Rastall, 2004). In addition to antimicrobial production, these microbes are highly capable of fermentation of dietary fibers. Therefore, Lactobacillus and Bifidobacterium are considered to ferment dietary fibers much more effectively than the pathogenic species in the gut (Hijova & Chmelarova, 2007). Increase in the amount of all short-chain fatty acids results in a decrease in the pH that indirectly affects the composition of the colonic microbiota, increases mineral absorption, decreases solubility of bile acids and reduces ammonia absorption (Radulian et al, 2009; Cook & Sellin, 1998) (Figure 2).

Inhibition of pathogen from causing infection: Another important function of the gut microbiota in the mammals is protection of gut from infection by pathogenic bacteria. During pathogen infection, three main functions may be seen:
Introduction

i) The gut microbiota might block the pathogen growth and thus interfere with the infection (Stecher & Hardt, 2011). ii) The microbiota might prime the host innate and adaptive immune system to prevent the development of infection and diseases, which is caused by the virulence factors of pathogen bacteria (Sekirov et al., 2010). iii) The gut microbiota eliminate the pathogens from the host gut at the end of the infection (Endt et al., 2010).

II.1.3. ROLE OF DIET ON GUT MICROBIOTA

Diet and different food habits are important factors in microbiota composition and development of mammals including the human being, either in the early life or in the late life. For instance, the diversity and composition of microbiota vary significantly between breast-fed and formula-fed infants in the early life. The intestinal microbiota of the breast-fed infants is more heterogeneous and more taxonomically diverse than the formula-fed infants (Schwiertz et al., 2010) with bifidobacteria as the key player in the gut microbial community of neonates (Turroni et al., 2012). The gut microbiota composition in the human and mammals is also under the influence of food habits. For instance malnutrition results in the lower abundance of Bacteroidetes in human gut microbiota, which are specialized for breaking down the carbohydrates in energy rich diet types (Ottman et al., 2012).

Carbohydrates form the bulk of most human and animal diets and are important nutrients for both host and microbiota. Mammals easily absorb simple sugars such as glucose and

Figure 2. The effects of human gut microbiota on host physiological processes. Arrows represent either stimulatory or inhibitory effects of gut microbiota on host physiological processes. Figure obtained from: http://www.nature.com/nrmicro/journal/v11/n4/full/nrmicro2974.html (Sommer & Bäckhed, 2013)
Introduction

galactose, as well as disaccharides and starch, in the proximal part of their small intestine (Ferraris, 2001). Yet, they are limited to hydrolyze and utilize more complex polysaccharides (Hooper et al, 2002). Therefore, a large quantity of undigested dietary carbohydrates including polysaccharides from plant cell wall components such as cellulose, xylan, pectin and fibrous substrates as well as undigested starch reaches the distal part of the gastrointestinal tract. This is where the gut microbiota play an important role by degrading these biomolecules (Hooper et al, 2002). Gut microbiota of most mammals and humans ferment these carbohydrate polymers that reach the distal gut and help extract nutrient value from otherwise poorly utilized dietary substrates. Therefore, the distal regions of the gut of several mammals are highly colonized with bacteria (Zoetendal et al, 2002). Gut microbiota hydrolyze these carbohydrates and consequently the hosts avoid the need to evolve the complex repertoire of glycosylhydrolases that would be required to break down the wide variety of linkages in dietary polysaccharides. The gut microbiota, in turn, gain access to a nutrient-rich environment, including complex dietary plant polysaccharides and host-derived glycans secreted on the surface of the epithelium and on the mucus layer, which is rich in carbohydrates (Corfield et al, 2001). Among these bacteria, bifidobacteria play the biggest role for carbohydrate fermentation in several mammals and humans (Ottman et al, 2012).

Proteins form a portion of diet of humans, carnivores and omnivores. Several bacteria use proteins as a carbon and nitrogen source and it has been demonstrated that protein-rich diets increase Clostridium populations (Zentek et al, 2004). The amount of protein that reaches the lower bowel may be consumed by gut microbiota, in which this amount depends greatly on the protein quality. Lower quality proteins, which are poorly digested, will provide more protein to microbes in the lower bowel. Therefore, either lower quality proteins or higher amounts of proteins can increase the occurrence of proteolytic bacteria such as Bacteroides (Wu et al, 2011). These bacteria can be pathogenic and/or produce putrefactive compounds such as ammonia, indoles, phenols, and sulphur-containing compounds. These compounds, which are regarded as detrimental for gut health in either humans or several animals, are toxic at high levels and are associated with several disease states (Backus et al, 2002) such as Inflammatory bowel disease (Pedersen et al, 2002; Tuohy et al, 2006) and chronic renal failure (Niwa, 2010).

According to the study conducted by Wu et al., short-term diet does not change the microbial composition in the human gut, while long-term diet has a tremendous role on modulating gut microbiome composition and enterotype partitioning (Wu et al, 2011). For instance, long term diet rich in animal protein, sugar, starch and fat and low in fiber, will result in Firmicutes and Proteobacteria dominance in the human gut microbiota. On the other hand, a diet type rich in vegetables and thus plant fibers, will result in the dominance of Actinobacteria and Bacteroidetes phyla (Wu et al, 2011; De Filippo et al, 2010).

Recent studies have shown that obese and lean people have different composition in their gut microbiota, specifically in the proportion of Bacteroidetes and Firmicutes. In obese people the Firmicutes are dominant while the Bacteroidetes are dominant in lean people (Ley et al, 2006). The Bacteroidetes degrades complex and otherwise indigestible dietary polysaccharides in the large intestine, resulting in the production of short-chain fatty acids,
Introduction

which are the energy source for the host (Tremaroli & Bäckhed, 2012; Thomas et al, 2011). In conclusion, the studies conducted to date about the role of diet on gut microbiota, endorse the fact that the intestinal microbiota in several mammals thrives on using polysaccharides and peptides, which are indigestible to mammals (Guarner & Malagelada, 2003).

II.2. TECHNIQUES FOR STUDYING DIVERSITY OF GUT MICROBIOTA

Several techniques are available for studying the diversity of gut microbiota in either humans or animals. Early experiments in this field used culture-based techniques, with a certain level of discriminatory power for identification of microorganisms (Temmerman et al, 2004). Most bacteria in the gastrointestinal tract are fastidious and hard to culture since they are anaerobic. Besides there is no uniform substrate preference for all of them (Inglis et al, 2012). Consequently only a fraction of the microorganisms in the gastrointestinal tract can be cultured and studied by culture-dependent techniques. Therefore, these techniques are limited in scope and lack precision for studying the diversity or functionality of microbiota, and the progress in this field was greatly hindered due to this fact (Kerr et al, 2013a).

The fact that 99% of the bacteria cannot be cultured or has not yet been cultured (Handelsman, 2004), leads the scientists to use culture-independent or molecular techniques, which is one of the most important developments of environmental microbiology since the 1980s (Keller & Zengler, 2004). These culture-independent techniques have several advantages over the culture-dependent techniques. Recent developments and rapid advances in molecular biology and sequencing have resulted in a more accurate understanding of the gut microbiota composition, the genetic elements of all the microorganisms in the gastrointestinal tract (microbiome), their dynamics, their interaction with the other gastrointestinal organs and the functionality of the intestinal ecosystem in human and many mammalian species (Ritchie et al, 2010). There are many molecular techniques of which phylogenetic clone libraries, phylogenetic microarray, community DNA profiling and metagenomics are most commonly used to study gut microbiota diversity (Lau & Liu, 2007).

Phylogenetic clone library is a sequencing-based technique that targets the 16S-rRNA gene, which is present and conserved among all members of a bacterial community, to study phylogenetic and bacterial community structures. In this technique, primers are used to amplify a portion of the 16S-rRNA gene, followed by ligation of the amplified region of the universal gene in bacterial plasmids. Afterwards, the plasmids are transformed into competent cells, such as E. coli, to generate a clone library (Inglis et al, 2012). The advantages are relative simplicity, representation of a single individual from the bacterial community by possessing a single insert in each clone; and the ability to near complete sequencing of 16S-rRNA gene (Inglis et al, 2012). On the other hand, the disadvantages are the underrepresentation of low abundance bacteria in the community, efficiency dependency on the quality of the inserts (Lovell et al, 2008) and the high expenses of sequencing as well. Despite all these aforementioned limitations, this method has still merit
Introduction

and has provided invaluable information about the bacterial community and composition of gut microbiota either in humans or mammals (Inglis et al., 2012) (Figure 3).

Phylogenetic microarray is a glass surface covered by thousands of covalently linked DNA probes, in which they will be hybridized by DNA or RNA. Subsets of probe sequences, which are specific to the ecological environment of interest, are required. The probes for studying the gastrointestinal microbial community are targeting only one or several parts of 16S-rRNA gene such as the V3 variable region (Claesson et al., 2009). This technique is widely used for monitoring gene expression, mutation in genomic DNA and DNA sequence polymorphism. Yet, it is not capable of detecting the unknowns, hybridization conditions are not optimal for all the hybrids and many probes are required for exploring the diversity (Gentry et al., 2006) (Figure 3).

Community DNA profiling is either sequence-dependent, such as Denaturing Gradient Gel Electrophoresis (DGGE) and Temperature Gradient Gel Electrophoresis (TGGE), or size-dependent electrophoresis such as Restriction Fragment Length Polymorphism (RFLP), Automated Ribosomal Intergenic Spacer Analysis (ARISA) and Single-Strand Conformation Polymorphism (SSCP).

In DGGE, a set of amplicons equal in size but different in sequence obtained from the 16S rRNA gene (Inglis et al., 2012), is applied to an polyacrylamide electrophoresis gel. The polyacrylamide gel has a denaturing agent including urea and formamide, so that the amplicons melts at various stages through the gel based on their GC content (Muyzer et al., 1993). The ability to excise and clone individual bands, is a significant advantage of DGGE analysis. Yet, visual identification of the bands in samples with complex banding patterns and high diversity can be difficult (Inglis et al., 2012) (Figure 3). TTGE is a form of electrophoresis that uses temperature to denature the sample as it moves across an acrylamide gel. Therefore, the amplicons are separated based on differences in melting temperature, sequence length, and GC content (Inglis et al., 2012). RFLP is a technique that uses fluorescently labeled PCR primers to amplify the gene of interest, followed by electrophoresis (Inglis et al., 2012). In order to study different environmental samples, ARISA can be used that uses PCR on the specific region of rRNA gene operon between 16S and 23S subunits (Ranjard et al., 2001). In SSCP electrophoresis is carried out to distinguish sequences fragments according to their different conformations (Hori et al., 2006).

Metagenomics is the study of genetic material recovered directly from environmental samples such as soil or water samples obtained from the environment. This technique enables studies of organisms that are not easily cultured in a laboratory, as well as studies of organisms in their natural environment (Sleator et al., 2008; Handelsman, 2004). This technique has been made possible by advances in next generation DNA sequencing technologies and bioinformatics, including sequencing the 16S-rRNA gene (Inglis et al., 2012) (Figure 3). Therefore, this technique has provided us with invaluable insight about the lifestyle and metabolic capabilities of the bacteria from environmental samples (Tringe et al., 2005). For instance, metagenomics allows to study the physiology and ecology of environmental microorganisms, novel genes and gene products, novel proteins and novel molecules with an antimicrobial activity (Handelsman, 2004). It also allows simultaneous
monitoring of a diversity of microorganisms and does not have the biases of DNA amplification. Yet, it is not possible to obtain finished genomes from all the microorganisms present in the sample, after performing assembly and annotation due to several reasons such as incompleteness of reference databases. This results in several gaps and misannotations in the chromosomes (Handelsman, 2004; Sleator et al, 2008). Particularly from less abundant members of the microbiota or in situations where a community contains many closely related species. Therefore, it is difficult to assign a function to specific species within one community (Sleator et al, 2008).

**Figure 3. Techniques used for studying the diversity of gut microbiota.** A) Phylogenetic clone library is a library in which the nucleotide sequences of interest are preserved as inserts to a plasmid that has been used to infect bacterial cells. B) Phylogenetic microarray is a high-throughput analytical method for microbial community analysis and consists of a glass covered with thousands of covalently linked DNA probes to which the DNA from the samples is added. C) Community DNA profiling is either “sequence dependent electrophoresis”, such as DGGE, TGGE and TGGE or “size dependent electrophoresis” such as RFLP, ARISA and SSCP. D) Metagenomics is the study of genetic material recovered directly from environmental samples, in which all community DNA is sequenced. This technique provides us not only diversity but also the functionality of gut microbiota. Figures derived from: [http://metagenomicsrevealed.yolasite.com/process-1.php](http://metagenomicsrevealed.yolasite.com/process-1.php) [http://www.envirologek.com/products_detail.php?indid=3&compid=15&cid=6&appid=&prodid=344&langid=1](http://www.envirologek.com/products_detail.php?indid=3&compid=15&cid=6&appid=&prodid=344&langid=1) [http://biointelligence.wordpress.com/microarray_research/](http://biointelligence.wordpress.com/microarray_research/)
II.3. CHEETAH (*ACINONYX JUBATUS*)

### II.3.1. CLASSES BASED ON DIETARY HABITS

Mammals can be divided into three general classes based on dietary habits, namely carnivores, herbivores and omnivores. Carnivores are animals that derive their energy and nutrients from mainly or exclusively animal tissues. They can either hunt other animals or scavenge animal bodies. Therefore, they have organs for capturing prey such as sharp teeth, strong jaws and claws (Gittleman *et al*, 2001). The cheetah (*Acinonyx jubatus*) is one of the 274 species in the mammalian order of Carnivora (Britannica, 2011), which is the only member of the genus *Acinonyx* (Treves & Karanth, 2003). This fast and relatively large animal has a deep chest and a relatively small head. It is also well-known by the tear marks running down its eyes in order to protect its eyes from sunlight and help this animal hunt (Hayward *et al*, 2006) (Figure 4).

Herbivores, such as deer and goat, consume plant material and the structure of their body and the inner organs is well adapted to eating only plants (Karban & Agrawal, 2002). They typically have wide flat teeth adapted to grinding the plant material like grass, tree bark and other tough plant material (Hummel *et al*, 2011) (Figure 4).

Omnivores, such as most bears as well as the human being, derive their energy and nutrients from sources that may include animals, plants, algae and fungi that possess teeth proper for consumption of various sorts of tissues such as plant and animal tissues (Deweerdt, 2011) (Figure 4).

![Image of skull and teeth structure in (a) carnivores, (b) herbivores and (c) omnivores.](http://avonapbiology2011-2012.wikispaces.com/Maya+Regalado)
II.3.2. VULNERABLE STATUS OF CHEETAHS

Cheetah, the world’s fastest mammal, is a predator carnivore. As a result these animals have limited metabolic ability to digest anything except proteins (Bradshaw et al, 1996). Cheetahs inhabit various places such as grassland savannahs and woodland habitats. They are listed as an endangered animal in the last decades and their distribution has declined dramatically in the wild (Nowell K., 1996). This is mostly due to the human actions such as destroying their natural habitat like much of Africa, Middle East and South Asia. Other human actions that further endanger this animal is hunting cheetahs and their prey such as gazelle and especially antelope, which is the most important prey for the cheetahs in the wild (O’Brien et al, 1985). Occasionally road accidents can cause danger to this animal as well (Williams, 2007). Based on an investigation in Zimbabwe, it was also found that several cheetahs have been killed in the last twenty years due to ingestion of anthrax infected meat, pneumonia, nephritis, asphyxiation, flea infection, accidental poisoning and fractures in their bones (Williams, 2007).

Cheetahs are mid-sized predators. Therefore, if there is not enough prey in the environment, they cannot compete for their food requirements with the larger predators such as lions, leopards, tigers and the African wild dogs. Obviously this unfortunate event can cause their starvation (Marker et al, 2003; Durant, 2004; Radloff & Du Toit, 2004; Hayward et al, 2006). It has also been seen and reported in the wild and also in the preserved areas that the cheetah cubs are preyed by other large predators such as lions and jaguars (Gros, 1998).

Lack of genetic variation, polymorphism and heterozygosity are other problems that may cause susceptibility and vulnerability of these endangered animals to specific diseases such as renal failure. Additionally, the level of variation in the aforementioned factors is higher in other mammals. Therefore, the fact that they compete with the other animals and survive in the wild, as well as adaptation to the ecological niche, is of great significance (O’Brien et al, 1983).

The increase in the habitat destruction of wild animals has led to the prospect of protecting these animals in captive breeding programs (O’Brien et al, 1985). In wildlife preservation, with the aim of protecting animals from extinction, conservation of threatened and endangered species is quite challenging. Captive breeding of cheetahs is very difficult to perform since a large number of the cubs die at birth due to disease susceptibility and maternal neglect (O’Brien et al, 1985; van Gelder, 1973).

Another problem that endangers cheetahs is the feeding mismanagement in the zoos that increases suboptimal health, gastrointestinal and metabolic diseases in these vulnerable animals. Additionally, improper feeding habits in captivity can lower the breeding performance in the cheetahs (Kotsch et al, 2002; Munson et al, 2005). Therefore, well-balanced diets can be a therapeutic intervention with representing an important route to prevent these feeding and breeding problems (Garcia-Mazcorro et al, 2011; Gaggia et al, 2010).
II.3.3. CHEETAH’S DIET AND ITS ROLE IN CHEETAH’S METABOLISM

Cheetahs are specialized in predation and have a protein-rich diet due to consuming mostly meat (Treves & Karanth, 2003). In the wild, these carnivores, generally prefer to capture and kill the most available medium-sized prey within a body mass range of 23-56 kg, such as antelope and gazelle (Nowak, 1999; Hayward et al., 2006). Though they are also able to utilize both larger and smaller prey (Mills et al., 2004). They mostly feed from the flesh of their prey, due to their relatively fragile teeth, skull and jaw (Van Valkenburgh et al., 1990). They also consume indigestible animal tissues such as small bones, tendons, skin, hair, and feathers derived from the prey (Depauw et al., 2011). These indigestible materials, which cannot be enzymatically digested in the body, have the potential to act as a substrate for microbial fermentation in the intestine of carnivores with the ability to produce high amounts of short-chain fatty acids. These short-chain fatty acids have numerous benefits on colonic function and metabolism such as energy source for colonocytes, stimulation of colonic blood flow and motility as well as decrease of the growth of pathogenic microbiota (Rondeau, 2003; Wong et al., 2006).

Wild cheetahs consume tendons, small bones and cartilage on a regularly basis. On the other hand, cheetahs in captivity are fed either raw meat diet supplemented with a vitamin and mineral premix or by a prey-based diet containing the whole carcasses, which has high amounts of indigestible animal tissues (Depauw et al., 2011). Therefore, a big difference between the diet of captive cheetahs and wild cheetahs is the nutrition composition of their diet. According to recent studies about the role of animal fibers on the cheetah gut microbiota and their health, the undigested part of an animal-based diet can be a source of short-chain fatty acids in the captive cheetahs and potentially other carnivores (Depauw et al., 2011). Moreover, the presence of animal fibers in the diet of captive cheetahs has a modifying effect on the bacterial fermentation in the intestine (Depauw et al., 2011). Therefore, it is of high importance to investigate whether the presence or absence of these indigestible animal tissues causes changes in intestinal fermentation. Moreover, the effect of prey-based diet type on the gut microbiota of cheetahs is promising due to the beneficial actions of the gut microbiota.

In general, the fermentation of protein sources is regarded as detrimental for gut health in either human or mammals. This process results in many by-products, such as ammonia, indolic and phenolic compounds. These compounds are toxic and have been linked to intestinal diseases e.g. inflammatory bowel disease (Pedersen et al., 2002; Tuohy et al., 2006) and chronic renal failure (Niwa, 2010), which is one of the causes of death in the captive cheetahs (Depauw et al., 2011). These putrefactive compounds have the potential to damage the gut ecosystem and influence the general metabolism of carnivores as they can be converted into toxic metabolites. To this end, several studies have been carried out, in which it has been observed that putrefactive compounds, namely indole, phenol and p-cresol, are higher when cheetahs are fed supplemented beef in comparison with cheetahs fed whole prey such as rabbit (Vester et al., 2008, 2009).
II.3.4. DOMESTIC CAT AS A MODEL FOR STUDYING CHEETAH GUT MICROBIOTA

Domestication is a continuous transition that differs by species, genes and environment, in which some characters may vary with circumstances (Price, 1984). In addition, it is reported that all domesticates manifest a considerable tolerance of proximity to people and also adaptations to a new diet. Domestication is not a single trait but a group of traits that consists of elements affecting emotion, mood and social communication, in which all have been modified in one way or another (Driscoll et al, 2009). The metabolic and morphological changes that result in behavioral adaptation to the human environment can lead to a remarkable dependence on humans for food and shelter, such as for domestic cats (Driscoll et al, 2009).

Patterns of sequence variation have been seen in the genome of domestic cats, which reflect a history of domestication and breed development (Driscoll et al, 2007). It is probable that the cat began its association with humans as a commensal, feeding on the rodent pests that infested the grain stores of the first farmers (Flood, 2001). It is generally considered that domestic cats have descended from the old world wildcats. Yet, they differ from wildcats in behavior, tameness, and coat color diversity (Peters et al, 2009).

The domestic cat has been proposed as a model for basic nutrients and energy requirements and feeding methods for captive felid populations such as the cheetah (Vester et al, 2008; Kerr et al, 2013b). It has been proposed by Dierenfeld et al. that nutritional requirements of domestic cats should form the basis of comparison in managed feeding programs for captive cheetahs (Dierenfeld, 1993). They both share several nutrition-related aberrations including food allergies, obesity and hyperlipidemia. Additionally, nutritionists and veterinarians can take blood samples from domestic cats, which is easier in comparison with the cheetahs. The blood samples are used to explore the blood metabolite concentrations, digestibility of food materials and intrinsic feline metabolism with specific nutritional requirements. Therefore, an extrapolation of dietary recommendations for domestic cats as well as their dietary profile, has been applied to other exotic felines like cheetahs (Lubbs et al, 2009; Vester et al, 2009).

It is not thoroughly scientifically applicable to extrapolate the data obtained from the domestic cats, including the data from dietary requirements and dietary profile, to the cheetahs. The behavioral, anatomical and nutritional characteristics of these two animals vary tremendously. For instance, domestic cats are frequently fed carbohydrate-rich extruded kibble diets (MacDonald et al, 1984; Zoran, 2002) while captive cheetahs are fed a meat-based or prey-based diet (Vester et al, 2009). Different sources of dietary fiber, such as pectin and cellulose, are present in domestic cats’ diet. Taxonomic and functional studies of the intestinal microbial communities have shown that domestic cats possess a well-developed complex microbial community which can also change depending upon their diet type (Lubbs et al, 2009; Vester et al, 2009). For instance, a higher protein level present in their diet has been associated with an increase in *Escherichia coli, Lactobacillus* spp., *Clostridium perfringens* and *Fusobacterium* spp. and a decrease in *Bifidobacterium* population in the fecal samples of cats (Lubbs et al, 2009; Vester et al, 2009). Moreover, *C. perfringens*, which has been identified as an intestinal pathogen in cheetahs (Citino, 1995),
Introduction

*E. coli* and *Lactobacillus* spp., were shown to increase when the cat’s diet was supplemented with pectin, whereas *Bifidobacterium* increased upon fructooligosaccharide supplementation (Barry *et al.*, 2012). Bermingham *et al.* showed that the diversity of the bacterial population in cats fed dry diets were lower compared with cats fed wet diets (Bermingham *et al.*, 2013). Moreover, it has been reported that cats fed a diet containing 4% pectin have a higher percentage of *Firmicutes* and *Spirochaetes* in comparison with cats that were fed a diet comprising 4% cellulose (Barry *et al.*, 2010).

Recently, it was reported by Tun *et al.* that *Bacteroidetes* is the most predominant (68%) bacterial phylum in the feline intestinal microbiome followed by *Firmicutes* and *Actinobacteria*, as it was found in the fecal microbiota of domestic cats (Khin *et al.*, 2012).

The current information about the gut microbiota of adult captive cheetahs has been obtained from only two captive cheetahs housed in Belgium. This information is collected in a clone library based on these two captive animals (Becker *et al.*, 2014). In order to build a clone library, fecal samples are collected and homogenization and community DNA extraction is carried out. Afterwards, 16S rRNA PCR is performed to prepare the clone library followed by two distinct steps. The first one is to perform V3-16S rRNA PCR, DGGE and data analysis. The second step is partial sequencing PCR followed by sequencing, assembly and annotation in order to build a phylogenetic tree by comparison against The Ribosomal Database Project II (RDP) (Figure 5). The group of sequences with ≤3% sequence divergence was regarded as an Operational Taxonomic Unit (OTU) or phylotypes. This phylogenetic tree works as a reference to identify the bacterial groups on the DGGE fingerprints of fecal samples under study.

Unexpectedly, *Bacteroidetes* and *Bifidobacteriaceae*, which can contribute in intestinal homeostasis, were not present in the 16S rRNA clone libraries of two Belgian captive cheetahs studied recently. Yet, a hardly detectable concentration of *Bacteroidetes* phylum was seen by qPCR. On the other hand, *Firmicutes* were in high number as the dominant group while the minority were *Actinobacteria*, *Proteobacteria* and *Fusobacteria*. Amongst the *Firmicutes*, *Clostridium* cluster XIVa, XI and I were in majority with 43%, 38% and 13% of all the bacteria respectively (Becker *et al.*, 2014).
Introduction

Figure 5. Constructing a clone library based on 16S rRNA gene. In order to build a clone library, fecal samples are collected followed by their processing to prepare the clone library. On one hand, sequencing, assembly and annotation are performed to eventually build a phylogenetic tree. On the other hand, V3-16S rRNA PCR is performed followed by DGGE to have the clones on the gel. The results obtained from both steps are used as a reference when analyzing the fecal samples under study.

To sum up, the current information about the gut microbiota of captive adult cheetahs is scarce. Consequently, more fecal samples should be collected from cheetahs housed in various zoos with different backgrounds and diet in order to eventually bring the correlation between diet, microbiota and health to light. By analyzing multiple fecal samples from these animals, more knowledge will be obtained about the diversity, composition and core microbiota of captive cheetahs.
CHAPTER III. GOALS

The cheetah is a carnivore that suffers from gastrointestinal disorders and suboptimal health in captivity, in which one reason can be due to an unbalanced diet. Diet, microbiota and health are strongly correlated. Diet has an effect on the gut microbiota since several nutritional components are enzymatically digested and absorbed by the host while the indigestible animal and plant tissues, such as complex carbohydrates, are fermented by the gut microbiota. This fermentation results in short-chain fatty acid production that benefits the host by decreasing inflammatory bowel disease, cardiovascular diseases, colon cancer and inflammation. In addition, gut microbiota benefit the host’s health through differentiation of the immune system, production of vitamins and tissue homeostasis. Therefore, gut microbiota should be taken into account when formulating the diet of captive cheetahs in order to improve their overall health status.

The relationship between diet and health of cheetahs in captivity has been explored in several studies. In contrast, the information about their gut microbiota remains scarce since the current knowledge about the fecal microbiota of adult captive cheetahs has only been obtained from a few animals from a single zoo. Therefore, in this study, we aim to collect more fecal samples from various cheetahs housed in different European zoos, with different genetic backgrounds, housing conditions and diets, in order to bring the fecal microbial composition of captive adult cheetahs to light. To this end, Denaturing Gradient Gel Electrophoresis (DGGE) and quantitative PCR (qPCR) will be used to further explore the fecal bacterial community composition.

The main goals of this Master thesis are:

- To describe the fecal microbial community composition of captive adult cheetahs housed in different European zoos. The goal is to investigate whether a core microbiota exists in the gut microbiota of captive cheetahs as well as to explore the potential impact of their diet, zoo and genetic backgrounds on overall gut microbiota composition. In order to perform DGGE, fecal samples should be homogenized followed by community DNA extraction. Afterwards, V3-16S rRNA PCR is performed to produce V3-16S rRNA amplicons for DGGE. Eventually, the data from the DGGE fingerprints will be analyzed with BioNumerics software followed by comparison of the fingerprints with the available clone library, previously built for the fecal samples of two captive adult cheetahs in Planckendael. By linking the clone library with the DGGE fingerprints, it will be possible to characterize the bacterial groups present in DGGE fingerprints of fecal samples.

- To quantify specific groups of bacteria in the fecal samples of captive adult cheetahs. This is carried out through qPCR for a prevailing group of bacteria in the clone library, namely *Clostridium* cluster XIVa. This technique quantifies *Clostridium* cluster XIVa and confirms the presence of this cluster in the DGGE fingerprints.
CHAPTER IV. RESULTS

IV.1. DGGE DATA

In this study, 55 fecal samples were collected from 36 captive cheetahs housed in 6 zoos, which were members of European Association of Zoos and Aquaria (EAZA). For each animal one sample was available and statistical randomization was used for animals with more than one sample to determine which samples to be included in this study. The zoos included Planckendael (PL), Ree Park Ebeltoft Safari (EB) and Réserve Africaine de Sigean (SI) located in Belgium, Denmark and France respectively and Gaia Zoo (GA), Safaripark Beekse Bergen (BB) and Zoo Parc Overloon (OV) in The Netherlands (Table 1). The goal was to characterize and compare the microbial community composition of the fecal microbiota of different animals from different zoos. To this end, DGGE fingerprinting was performed based on the V3 region of the 16S rRNA gene obtained from V3-16S rRNA PCR.

<table>
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<th>Name</th>
<th>Sex</th>
<th>Year of Birth</th>
<th>Housing</th>
<th>Parents</th>
<th>Sample code</th>
<th>DNA code</th>
<th>Diet</th>
<th>Diet type</th>
<th>Dietary Regime</th>
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Table 1: Animal Information

- **Zoo**
  - Planckendael in Belgium
  - Gaia Zoo in The Netherlands
  - Safaripark Beekse Bergen in The Netherlands
  - Zoo Parc Overloon in The Netherlands

- **Name**
- **Sex**
- **Year of Birth**
- **Housing**
- **Parents**
- **Sample code**
- **DNA code**
- **Diet**
- **Diet type**
- **Dietary Regime**
## Results

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<td>2010</td>
<td>Sire: 205011 Dam: 2010046</td>
<td>FR2-SI-PC-T1 CH464 Chicken</td>
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<td>Sire: 576 Dam: 1041</td>
<td>FR3-SI-PC-T1 CH473 Chicken</td>
<td>A</td>
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<td></td>
</tr>
<tr>
<td>Nuru M</td>
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<td>2005</td>
<td>Sire: 041104 Dam: 041077</td>
<td>DK1-EB-P-T1 CH440 Rabbit</td>
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<td>2009</td>
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<td>Aduke F</td>
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<td>DK8-EB-P-T1 CH448 Chicken</td>
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<td>Suna F</td>
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<td>Hurley M</td>
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<td>Jack M</td>
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<td>2010</td>
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<td>DK11-EB-P-T1 CH453 Rabbit</td>
<td>A</td>
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</table>

Table 1. Metadata of all the animals in this study. Dietary regimen is defined as the diet an animal has in a specific period of time while diet is defined by the animal that the cheetahs are fed, such as antelope, chicken or horse. Diet type has two categories in this study which is prey and meat. A: Prey, which is the whole carcass with variable amounts of indigestible animal tissues, such as raw bones, tendons, cartilage, skin, hair or feather. B: Chunked raw meat supplemented with vitamin and mineral premix. 1: Horse meat supplemented with Carnicon vitamins and minerals. These animals are sometimes fed rabbit and they have fasting days with less amounts of meat. 2: Minced meat and chopped meat with water, Royal Canin Renal chunk, Pancrex and Prineral 20mg for the “Kivili”. “Jinsi” receives the same diet with addition of Ipakitine, Gistocal and blood. “Sawa” receives meat, Gistocal, water and blood. 3: Rabbit and doves as prey. These animals are sometimes fed meat supplemented with minerals and vitamins. 4: Rabbit as prey and they are...
Results

sometimes fed horse and beef meat. They have a fasting day on Thursdays. 5: Whole chicken as prey, which are freshly killed. These cheetahs have a fasting day on Mondays. 6: Chicken without a head or rabbit as a whole prey.

I: Outside is grass and indoor is concrete. These two brothers always live together and their sleeping beds are made of straws. II: Concrete inside with a bed of straw, they each have their separate inside house, grassland outside which they share. III: Inside is concrete and they sleep separately. Outside is covered with grass and is for running and (females and males are separate). IV: Grass outside. The males stay together while females are separate. Inside is covered with sand and concrete sometimes. V: Different places for females and males in the zoo. Females live in the enclosure. VI: Nuru, Abayomy, Aduke and Richard live alone while Split and Izzy live together in the concrete inside. Hollander, Hurley and Jack share the outside grass enclosure together.

IV.1.1. ANALYSIS OF ALL DGGE FINGERPRINTS

Clustering the fingerprints: The DGGE fingerprint profiles obtained from 55 fecal samples were compared by hierarchical clustering using the Pearson correlation coefficient (Figure 6), as well as the Dice correlation coefficient (Figure 7). Pearson correlation coefficient is based on the intensity of the bands whereas Dice correlation coefficient is based on the presence or absence of bands in the fingerprints. The analysis of the DGGE fingerprints showed variation in intensity of bands between the animals, which were labeled by zoo, diet and diet type. No zoo-specific or diet type-specific clustering was observed in the DGGE profiles. Yet, several smaller clusters were obtained that were not entirely correlated with zoo or diet type (see pages 37 and 38).

Pairwise comparison of all the fingerprints: The fingerprints were compared in a pairwise manner with the Pearson correlation coefficient, which resulted in a data set of 1540 pairwise combinations. The Pearson correlation coefficient was chosen over Dice since intensity of the bands was more important than only their presence, in order to study the predominant bacteria. Moreover, the boxplots made for pairwise similarities between fingerprints per zoo or per diet type did not differ drastically from Pearson to Dice.
Figure 6. Clusters obtained from all fingerprints based on Pearson correlation coefficient. The fingerprints were clustered according to Pearson correlation coefficient. The numbers on the branches indicate the similarity percentages. Dark green: SI - Blue: EB – Red: OV – light green: GA – Orange: BB – Light blue: PL
Figure 7. **Clusters obtained from all fingerprints based on Dice correlation coefficient.** The fingerprints were clustered according to Dice correlation coefficient. The numbers on the branches indicate the similarity percentages. Light green: GA – Red: OV – Blue: EB – Orange: BB – Light blue: PL – Green: SI
Results

Pairwise comparison of the fingerprints per zoo: The variation of the predominant bacterial diversity within the 6 zoos was assessed by determining pairwise similarity values, according to the Pearson correlation coefficient, between all fecal fingerprints in each group. Further comparison was done by using boxplot analysis per zoo. As depicted in (Figure 8), the highest variability between the fingerprints per zoo was observed in the DGGE profiles in Réserve Africaine de Sigean and Zoo Parc Overloon, while the lowest variability was observed between the samples from Planckendael.

Figure 8. Boxplot for pairwise comparisons of the DGGE fingerprints per zoo based on Pearson correlation coefficient. The DGGE fingerprints of fecal samples grouped in a zoo were compared in a pairwise manner followed by boxplot analysis. PL: Planckendael GA: Gaia Zoo OV: Zoo Parc Overloon BB: Safaripark Beekse Bergen EB: Ree Park Ebeltoft Safari SI: Réserve Africaine de Sigean

Pairwise comparison of the fingerprints per diet type: The variation of the predominant bacterial diversity in the animals fed meat versus prey was assessed by determining pairwise similarity values, according to the Pearson correlation coefficient, between all fecal fingerprints in each group. Samples were further compared by boxplot analysis (Figure 9). The boxplot analysis revealed that without taking the number of samples into account, the fingerprints from cheetahs fed meat had less variability compared to the animals fed prey. Yet, the median was the same.
Results

Pairwise comparison of the fingerprints per animal: The variation of the predominant bacterial diversity within each animal, with more than one sample, was assessed by determining pairwise similarity values, according to Pearson correlation coefficient. The samples were further compared by boxplot analysis per animal (Figure 10). A trend was visualized in the boxplots that 75% of these animals had more than 80% similarity between their fingerprints form the fecal samples. The remaining 25% had more variability in their fingerprints.
Results

Figure 10. Boxplot for pairwise comparisons of the DGGE fingerprints per animal, with Pearson correlation coefficient. The fingerprints from each animal, with more than 1 fecal sample, were compared in a pairwise manner. The animals with one fecal sample were excluded from this study. Animals FR5, FR3 and DK3 had 3 fecal samples, while the remaining had 2 samples.
Results

IV.1.2. BAND RICHNESS OF THE DGGE FINGERPRINTS PER ZOO

The bands from the fingerprint profiles were assigned to band-classes depending upon their position in the fingerprint. Following, these band-classes were correlated to the clones in the clone library. As a result, a total of 42 band-classes were assigned. The band richness, which is the number of bands per fingerprint, was determined for each DGGE profile in order to assess the variability and richness of the intestinal ecosystem of these animals. The lowest variability in band richness belonged to the fingerprints of cheetahs from Gaia Zoo, while the highest variability of band richness belonged to the fingerprints from Safaripark Beekse Bergen (Table 2).

<table>
<thead>
<tr>
<th>Zoo</th>
<th>Average of band richness</th>
<th>Range of band richness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Planckendael</td>
<td>20</td>
<td>14-23</td>
</tr>
<tr>
<td>Réserve Africaine de Sigean</td>
<td>17</td>
<td>9-23</td>
</tr>
<tr>
<td>Safaripark Beekse Bergen</td>
<td>19</td>
<td>13-25</td>
</tr>
<tr>
<td>Zoo Parc Overloon</td>
<td>17</td>
<td>13-22</td>
</tr>
<tr>
<td>Gaia Zoo</td>
<td>11</td>
<td>9-12</td>
</tr>
<tr>
<td>Ree Park Ebeltoft Safari</td>
<td>16</td>
<td>12-24</td>
</tr>
</tbody>
</table>

Table 2. The average and range of band richness per zoo.

Also, the band richness of DGGE fingerprints per zoo was analyzed in boxplots (Figure 11). A large variability was seen in fingerprints from OV and SI, whereas fingerprints from GA had the least variability among the zoos. Moreover, the mean of band richness was lower in fingerprints from GA compared with the other zoos.

Figure 11. Boxplot analysis of band richness of the DGGE fingerprints per zoo. The number of bands of DGGE fingerprints, which were grouped per zoo, was calculated followed by boxplot analysis. Outlier values are indicated by open circles and stars.

PL: Planckendael
GA: Gaia Zoo
OV: Zoo Parc Overloon
BB: Safaripark Beekse Bergen
EB: Ree Park Ebeltoft Safari
SI: Réserve Africaine de Sigean
Results

In order to visualize the band-classes present in the fingerprints, a histogram was generated (Figure 12). It was depicted in the histogram that several band-classes such as 50.1%, 54.3% and 67.5% were present in more than 70% of the fingerprints, whereas, band-classes 39.7%, 47.4% and 80.8% were present in more than 60% of the DGGE profiles. Several band-classes were not abundant in these fingerprints, which had less than 10% occurrence, such as band-classes 55.5% and 66.2% (Figure 12). Therefore, it can be deduced that the more prevailing fingerprints such as band-class 54.3%, correlating to *Clostridium* cluster XIVa, may be a member of the core microbiota in these samples.

![Figure 12. Band-classes present in the fingerprints. The percentage of fingerprints that possesses each band-class.](image)

In order to document the percentage of fingerprints per zoo, that contained a particular band-class, a table was generated (Table 3). It is illustrated in this table that band-classes 54.3% and 67.5% were present in all the 6 zoos.
### Table 3. The percentages of fingerprints per zoo, containing each band-class. For each band-class, the percentage of fingerprints per zoo was calculated.

<table>
<thead>
<tr>
<th>Band-class %</th>
<th>PL</th>
<th>GA</th>
<th>BB</th>
<th>OV</th>
<th>EB</th>
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<tr>
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</table>
Results

IV. LINKING THE DGGE DATA WITH THE CLONE LIBRARY

IV.2. COMMON BAND-CLASSES IN THE FINGERPRINTS

To bring to light the common bacterial diversity in the fecal samples of these cheetahs, we focused on 3 clusters to which most phylotypes of the clone libraries were assigned to, which might be members of the core microbiota in these 55 fecal samples.

*Clostridium cluster XIVa:* The DGGE fingerprints were compared with the clone library previously constructed from the fecal samples of 2 cheetahs in Belgium. According to this comparison, all the fingerprints consisted of band-classes 50.1% and 54.3%, with different intensities. The Operational Taxonomic Units (OTUs) correlating to these band-classes were OTU 21, 10, 7 and 9 from the clone library, representing *Clostridium cluster XIVa.* Moreover, other band-classes, namely 47.4%, 49.1% and 53.3%, correlated as well to OTUs representing this cluster. However, latter band-classes were only present in some of the fingerprints (Figure 13).

*Clostridium cluster XI:* All the fingerprints had a band assigned to either band-class 80.8% or 81.6%, with different intensities. OTUs 3 and 4 correlated to these band-classes that represented *Clostridium cluster XI* in the clone library (Figure 13).

*Clostridium cluster I:* Band-classes 67.5% and 68.2%, with low intensity, were present in 70% of the fingerprints, representing *Clostridium cluster I.* Due to the limitations of DGGE such as difficulty in visualization, this band-class might be present in almost all the fingerprints. The OTU, correlating to these band-classes, was closely related to *Clostridium perfringens* (Figure 13).
Results

Figure 13. *Clostridium* clusters XIVa, XI and I in the fingerprints. *Clostridium* cluster XIVa and XI were present in all the fingerprints whereas *Clostridium* cluster I was present in 70% of the DGGE profiles. There were 5 band-classes representing *Clostridium* cluster XIVa in the fingerprints. The clustering was based on Pearson correlation coefficient. The numbers on the branches indicate the similarity percentages.

### IV.2.2. LINKING BAND-CLASSES WITH FINGERPRINT CLUSTERS

Clusters based on Pearson correlation coefficient: Pearson correlation coefficient clustering of all fingerprint profiles resulted in two complete separate clusters with 34.5% similarity (Figure 6). The first cluster consisted of 5 fingerprints from cheetahs in Réserve Africaine de Sigean and was separated from the rest mostly due to band-classes 28.3% and 41.3% (Figure 14). These band-classes did not correlate to any clones in the clone library. After excising and sequencing these bands, it was revealed that they both belonged to *Lactobacillus* spp. The OTUs 15, 26 and 42 correlated to this genus in the clone library as well.
Results

Compared to the other animals, these 5 animals were the only ones on a chicken only dietary regimen.

![Figure 14. Band-classes 28.3% and 41.3% were distinct in 5 fingerprints belonging to Réserve Africaine de Sigean. The clustering was based on Pearson correlation coefficient. The numbers on the branches indicate the similarity percentages.](image)

Two clusters were obtained within the remaining 49 fingerprints with 55.4% similarity due to the band intensity in band-classes 50.1% and 54.3%, correlating to *Clostridium* cluster XIVa. One of these clusters consisted of 14 fingerprints with low intensity in these 2 band-classes, while the other cluster, containing 35 fingerprints, had relatively more intense bands in these two band-classes (Figure 6).

Fingerprint obtained from the fecal sample of NL12-OV-M-T13 clustered separately from 49 fingerprints with 45.5% similarity. This animal had a distinct band in band-class 31.4% that does not correlate with any of the clones in the clone library. Yet, it displayed bands correlating to *Clostridium* cluster XIVa, XI and I as all the other fingerprints do (Figure 6).

According to Pearson correlation coefficient, the profiles of two animals from the same zoo and on the same diet, namely DK8-EB-PC-T1 and DK7-EB-PC-T1, clustered with 99.3% similarity together. These profiles had very intense bands for *Clostridium* clusters XIVa, XI and I and the same band-classes (Figure 15).

![Figure 15. The 2 fingerprints clustered together with 99.3% similarity based on Pearson correlation coefficient. These two fingerprints were obtained from 2 animals in Ree Park Ebeltoft Safari. The clustering was based on Pearson correlation coefficient. The numbers on the branches indicate the similarity percentages.](image)

Clusters based on Dice correlation coefficient: Based on Dice correlation coefficient, 2 separate clusters were obtained from all fingerprints with 50.3% similarity. One of these clusters consisted of only two fingerprints, namely NL13-GA-M-T1 and NL15-GA-M-T1 from Gaia Zoo, with 55.6% similarity (Figure 7). A cluster with 6 fingerprints was made with 68.7% similarity, in which all profiles belonged to animals fed chicken in Réserve Africaine de Sigean. They all had band-classes 28.3% and 41.3% in common, which did not correlate to any clones in the clone library (Figure 16), which belonged to *Lactobacillus* spp. after sequencing. This data confirmed the clustering based on the Pearson correlation coefficient.
Results

**Figure 16.** The 6 fingerprints from fecal samples of animals in Réserve Africaine de Sigean. These profiles have 2 band-classes, not correlating to the clones in the clone library. The clustering was based on Pearson correlation coefficient. The numbers on the branches indicate the similarity percentages.

According to Dice correlation coefficient, two animals from different zoos and on different dietary regimes, namely NL9-OV-M3 and B2-PL-M-T10, clustered with 95% similarity together. Their fingerprints shared 90% of their band-classes (Figure 17).

**Figure 17.** Fingerprints from two animals from different zoos and on different diets with 95% similarity. Animals NL9-OV-M3 and B2-PL-M-T10 belonged to different zoos and they had different diets. Yet, the fingerprints from their fecal samples were 95% similar. The clustering was based on Pearson correlation coefficient. The numbers on the branches indicate the similarity percentages.

There were no band-classes specifically associated with the profiles in BB, SI or GA zoos, whereas it was the case for fingerprints from PL, OV and EB.

**Band-classes in Planckendael:** The fingerprints from Planckendael displayed more than 50% similarity to one another. They also displayed more intense bands for band-classes 67.5% and 68.2%, correlating to *Clostridium* cluster I, in comparison with the other fingerprints. Band-classes 28.3% and 29.2%, which had very low intensity, were present in all 4 DGGE fingerprints of the animals in Planckendael. They did not correlate to any of the clones present in the clone library (Figure 18).

**Figure 18.** Band-classes 28.3% and 29.2% were present in the fingerprints from animals housed in Planckendael. These band-classes did not correlate to any of the clones in the clone library. The clustering was based on Pearson correlation coefficient. The numbers on the branches indicate the similarity percentages.
Results

Band-classes from Ree Park Ebeltoft Safari and Zooparc Overloon: The fingerprints from Ree Park Ebeltoft Safari and Zooparc Overloon had all the 5 band-classes correlating to Clostridium cluster XIVa (47.4%, 49.1%, 53.3%, 50.1% and 54.3%) while the other zoos displayed only 1-4 of these band-classes, which varied among zoos (Figure 19).

Figure 19. The fingerprints of the fecal samples from Ree Park Ebeltoft Safari and Zoo Parc Overloon had more band-classes correlating to Clostridium cluster XIVa. There were 5 band-classes, namely 47.4%, 49.1%, 50.1%, 53.3% and 54.3%, correlating to Clostridium cluster XIVa in these fingerprints. The clustering was based on Pearson correlation coefficient. The numbers on the branches indicate the similarity percentages.
Results

IV.3. QUANTITATIVE-PCR RESULTS

Clostridium cluster XIVa, present in all the DGGE fingerprints, was quantified by qPCR in order to confirm the DGGE fingerprints and to link their quantity to the intensity of the bands, correlating to this cluster.

Clostridium cluster XIVa, was present in all the DNA samples except for NL14-GA-M-T1. This cluster was not detectable in this sample. Yet, its DGGE fingerprint had very low intense bands in band-classes 47.4% and 50.3%, correlating to this cluster. The variability of the quantity of this cluster was between 3.04E+06 CFU/g and 1.13E+11 CFU/g in the feces, which belonged to FR6-SI-PC-T2 and B2-PL-M-T10 respectively.

VI.3.1. THE QUANTITY OF CLOSTRIDIUM CLUSTER XIVa PER ZOO

The quantity of Clostridium cluster XIVa was calculated for each sample followed by boxplot analysis per zoo (Figure 20) in order to see the quantitative variability of Clostridium cluster XIVa per zoo. It was illustrated by the boxplot that the highest variability of Clostridium cluster XIVa belonged to samples from SI, whereas the lowest variability belonged to OV.

Figure 20. Boxplot analysis for quantification of Clostridium cluster XIVa per zoo. The calculated amount of this cluster is depicted by boxplot per zoo. The biggest variability is seen in Gaia Zoo, while the smallest variability is seen in Zooparc Overloon. The outliers are indicated by open circles.

PL: Planckendael
GA: Gaia Zoo
OV: Zoo Parc Overloon
BB: Safaripark Beekse Bergen
EB: Ree Park Ebeltoft Safari
SI: Réserve Africaine de Sigean
VI.3.2. THE QUANTITY OF CLOSTRIDIUM CLUSTER XIVa PER ANIMAL

A histogram was assessed for the quantity of Clostridium cluster XIVa in the fecal sample of all animals (Figure 21). Among 36 animals in this study, 16 animals had more than 1 fecal sample; therefore, the mean quantity of Clostridium cluster XIVa was calculated for these 16 animals. The highest quantity belonged to animal B2 with 2 fecal samples, while Clostridium cluster XIVa was not detectible in animal NL14.

![Histogram of quantity of Clostridium cluster XIVa per animal.](image)

**Figure 21.** Histogram of quantity of Clostridium cluster XIVa per animal. As depicted, the highest value belonged to animal B2, whereas Clostridium cluster XIVa was not detectable in animal NL14. Among the animals, 16 animals had more than 1 sample. Animals FR2, DK1, FR4, FR1, NL11, B1, B2, DK2, DK7, NL9, NL7 and NL10 had 2 fecal samples while animals DK3, FR3 and FR5 had 3 samples. The rest had one fecal sample in this study.

VI.3.3. CLOSTRIDIUM CLUSTER XIVa VERSUS THE DGGE BAND INTENSITY

In order to verify whether the DGGE band intensity of the band-classes, correlating to Clostridium cluster XIVa, correlate with their quantity in each sample, a scatter plot was generated (Figure 22). It was depicted in the scatter plot that with an increase in the intensity of DGGE bands, the CFU/g did not change in an ascending pattern. This result illustrates that the band intensity in the DGGE profiles is not proportional to the concentration of Clostridium cluster XIVa determined by qPCR.
Results

Figure 22. The scatter plot of the intensity of band-classes, correlating to Clostridium cluster XIVa, against its quantity in each sample. The mean intensity of band-classes 47.4%, 49.1%, 50.1%, 53.5% and 54.3%, which correlate to Clostridium cluster XIVa, was plotted against the absolute quantity of this cluster obtained from qPCR.
Discussion

CHAPTER V. DISCUSSION

This study set out to describe the fecal microbial community composition in 55 fecal samples collected from captive adult cheetahs housed in 6 different European zoos. To this end, denaturing gradient gel electrophoresis (DGGE) was performed followed by comparison of the DGGE fingerprints with the available clone library, which was previously made from fecal samples of two cheetahs in Belgium (Becker et al., 2014). In addition to DGGE profiling, this study included quantitative PCR (qPCR) with the aim to quantify *Clostridium* cluster XIVa, which is one of the most prevailing bacterial groups in the clone library.

In the current study, DGGE fingerprinting on the cross-sectional data set gave rise to different community profiles. The DGGE fingerprinting revealed a largely animal-specific clustering and indicated a clear difference in community structure in the fecal samples. This inter-animal variation has also been shown in mice (Hildebrand et al., 2013) and cats (Desai et al., 2009) and many other mammals including humans (Knight et al., 2012). This study also revealed that the microbiota is affected by a combination of factors including age, sex, diet, diet type, genetic background and housing of the captive adult cheetahs. Other studies have also revealed that factors such as age, genetics, environment, diet and drugs, might play a role in the DGGE fingerprints of humans as well (Wu et al., 2011; Knight et al., 2012). For instance, it was shown that by intake of antibiotic, the band richness in the DGGE profiles might decrease in the patients with cystic fibrosis as well as result in an increase or decrease in the intensity of some bands (Duytschaever et al., 2011). It was also shown that the mice, which were born and raised in the same housing, showed little variation in their microbiota profiles compared to the mice in different housings. It was also revealed that their gut microbiota composition changed by changing their housings (Spor et al., 2011). Yet, the degree of the divergence in their gut microbiota composition depended on the genotype of the mice (Deloris Alexander et al., 2006).

By clustering the fingerprints according to the Pearson and Dice correlation coefficients, no diet type or zoo specific clustering was visualized since these factors do not solely contribute to the band patterns. These factors, as well as many other environmental factors, may have contributed to the observation that the fingerprints were unique, which indicates large inter- and intra-individual variations (Duytschaever et al., 2013). Yet, a separate cluster was visualized by Pearson correlation coefficient with 5 fingerprints from SI as well as a separate cluster with 6 fingerprints from SI with Dice correlation coefficient. Both clusters were obtained mainly due to band-classes 28.3% and 41.3%, which belonged to *Lactobacillus spp.* after band excision and sequencing.

The number of bands per fingerprint and per zoo was studied and analyzed, which revealed a lower mean and range of band richness in the 3 fingerprints from Gaia Zoo. These 3 animals had consumed beef meat with renal disorder sustaining supplements such as Ipakitine and Gistocal. Moreover, it has been shown in other studies that beef meat is digested mostly by the host (Vester et al., 2010) and leaves less substrates for the gut microbiota to ferment (Niba et al., 2009). Therefore, more fecal samples should be collected and analyzed from these animals in Gaia Zoo to substantiate the diversity in their gut microbiota. Band richness is considered to be a valuable parameter for measuring the intestinal ecosystem’s fitness and flexibility (Wittebolle et al., 2009). The fewer bands
Discussion

present in a fingerprint, the more negative impact it has on the ecosystem’s functional stability because it leads to lower productivity as well as less flexibility to compensate for natural fluctuations (Yachi & Loreau, 1999).

All DGGE profiles had band-classes correlating to Clostridium cluster XIVa and XI, which indicate that they are part of the core microbiota in these animals as it was also shown for 2 captive cheetahs in Planckendael (Becker et al, 2014). Among the fingerprints, 70% of the samples displayed band-classes correlating to cluster I. These samples did not have any factors, such as diet type or zoo, in common. This visualization can either be precise or can be due to the limitations of DGGE in band visualization (Inglis et al, 2012). The band-classes present in most of the zoos, might also be members of core microbiota in these fecal samples but might not be detected by DGGE due to technical limitations. DGGE fingerprinting is an efficient technique to study the fecal microbial community composition of captive adult cheetahs as well as other animals and humans (Inglis et al, 2012). The ability to excise and clone individual bands, is a significant advantage of DGGE analysis. However, multiple taxa can occupy the same position in the gel (Muyzer et al, 1993; Muyzer & Smalla, 1998), which results in an under-estimation of diversity. Moreover, multiple DGGE bands can also correlate to a single taxon since a species can contain multiple 16S rRNA genes (Acinas et al, 2004), which results in an over-estimation of abundance and/or diversity (Inglis et al, 2012). Therefore, visual identification of the bands in samples with complex banding patterns and high diversity can be difficult (Inglis et al, 2012). Moreover, the steps before performing DGGE and the quality of the gel may lead to lack of visualization of the bands (Inglis et al, 2012). In order to address some of these disadvantages, other techniques can be used in the future in order to substantiate the results obtained from DGGE (Inglis et al, 2012).

Since the fecal samples were homogenized and community DNA was extracted from the homogenate (Pitcher et al, 1989), it is always possible that the DNA has impurity, low quality or degradation as well as loss of DNA. Therefore, the DNA quality and quantity should always be checked prior to performing V3-16S rRNA PCR and DGGE. Homogenization, DNA extraction and PCR may bring biases, which are inevitable. Occasionally, they lead to an increase or decrease in the intensity of some bands on the DGGE fingerprint profiles (Ariebenjohan et al, 2010; Claassen et al, 2013).

The presence of Clostridium cluster XIVa, which made up to 43% of the 702 clones in the clone library (Becker et al, 2014), was confirmed in all the fecal samples by qPCR, except for one animal. On one hand, qPCR is not capable of detecting all the bacterial cells present in the fecal sample, as for the majority of techniques (Inglis et al, 2012). On the other hand, there is a possibility that inaccurate measurement take place due to nonspecific amplification if the amplicons and the primers specificity are not checked properly. This results in overestimation of cell densities in the samples (Inglis et al, 2012). One of the major advantages of qPCR is the fact that it has relatively high throughput and it is simple and fast after developing the primers and the PCR program (Inglis et al, 2012). Clostridium cluster XIVa, which is a phylogenetically heterogeneous cluster, consists of gram-positive bacterial groups in the Firmicutes phylum. The cluster consists of several families such as Ruminococcaceae, which are the representative of Clostridium cluster XIVa in several phylogenetic clone libraries (Hoskins, 1993). Blautia is one of the members of this cluster.
Discussion

(Park et al, 2012), which produces short-chain fatty acids such as butyrate as a source of energy for colonic epithelial cells. The members of this genus constitute about $10^{10}$–$10^{11}$ bacteria cells per gram in the human colon (Hayashi et al, 2006). This genus can also produce lactate and acetate as the major end product of carbohydrate fermentation in the human gut (Park et al, 2013). In addition, most members of this genus do not produce indole as a putrefactive compound (Park et al, 2012; Willems & Collins, 1995), which is regarded as detrimental for gut health in either humans or several animals. This compound can be associated with several disease states (Backus et al, 2002) such as chronic renal failure (Niwa, 2010).

Clostridium cluster XIVa was not detectable in the fecal sample of animal NL14-GA-M-T1, though it had vague bands on its DGGE fingerprint correlating to this cluster. This animal suffered from renal disorder and it was on a special diet including supplements Ipakitine and Gistocal (Table 1). It has been reported in cats and dogs that inflammatory bowel diseases, which are associated with damaged mucus layer, can be associated with reduced proportion of Clostridium cluster XIVa as well (Inness et al, 2007). Moreover, the decrease in this cluster results in a decline in butyrate production under anaerobic condition (Eeckhaut et al, 2010). This metabolite has important biological functions such as providing energy for colonocytes, maintenance of the gastrointestinal barrier, elicitng an anti-inflammatory response, as well as being involved in prevention of colorectal cancer (Hamer et al, 2008; Canani et al, 2011). Therefore, the renal disease might be among the reasons for the decreased proportion of Clostridium cluster XIVa in this animal in Gaia Zoo (Niwa, 2010). Yet, the gut microbial ecosystem has a broad phylogenetic spectrum of bacteria that are capable of producing butyrate. Consequently, it is not clear to which extent the reduction of these bacterial groups may have health consequences (Collins et al, 1994).

Clostridium cluster XI, present in all the DGGE fingerprints in the current study, accounts for 22% of the fecal microbiota in healthy cats (Ritchie et al, 2010) and many members of this genus produce short-chain fatty acids from mono- and disaccharides (Kitahara et al, 2001). Clostridium hiranonis is one of the representatives of this cluster and was first described in human feces (Kitahara et al, 2001) and was also detected in the 2 captive cheetahs in Planckendael (Becker et al, 2014).

In the future, more fecal samples from captive adult cheetahs can be collected in order to understand the fecal microbial community composition of captive adult cheetahs in detail as well as to study its diversity. Moreover, in a longer period of time, temporal stability can be studied in the gut microbiota of captive cheetahs, as it has been performed for several animals such as mice (Dimitriu et al, 2013). In order to better understand the DGGE profiles and the present bacterial groups, more zoomed DGGE can be performed to better visualize the profiles with complex banding patterns and high diversity. Furthermore, the bands belonging to band-classes, which do not correlate to the clones in the clone library, can be excised and sequenced to bring the bacterial composition to light. In addition to the description of the diversity of gut microbiota of captive cheetahs, the functionality of the bacterial groups should be studied as well to understand the role of the members of the gut microbiota in captive cheetahs. For the band-classes present in most of the zoos group-specific qPCR can be performed in order to quantitatively determine all the members of the core microbiota in the captive adult cheetahs.
CHAPTER VI. CONCLUSION

This study aimed to characterize the fecal microbial community composition of captive adult cheetahs housed in several European zoos. To this end, DGGE was performed based on the V3 region of the 16S rRNA gene, which revealed complex banding patterns and distinct bacterial community. Next, the fingerprints were compared with the available clone library from 2 captive cheetahs in Belgium. This study revealed a complex microbial diversity in which Firmicutes were dominant. Among the Firmicutes, Clostridium cluster XIVa and XI were present in all the fingerprints whereas cluster I was present in 70% of them.

According to data analysis, neither diet type nor zoo specific clustering was visualized in the fingerprints. This indicates that the gut microbiota composition is affected by a combination of factors as it is for humans (Knight et al., 2012). Clostridium cluster XIVa and XI were present in all the DGGE profiles, while cluster I was visualized in 70% of the fingerprints, which might have been due to the limitations of DGGE in band visualization (Inglis et al., 2012). Therefore, these 3 clusters of Clostridium might be part of the core microbiota of these 55 fecal samples as it has been shown in 2 captive cheetahs in Planckendael (Becker et al., 2014).

The boxplot analysis of the fingerprints per animal, per zoo and per diet type revealed the variation of the predominant bacterial diversity between the fingerprints. It was illustrated by boxplot analysis of the band richness per zoo that the mean and range of band richness was less in Gaia zoo compared to the other zoos. The biggest mean of band number belonged to Planckendael since they had more bacterial variety in their fingerprints. In addition, the highest variability of band richness belonged to Safaripark Beekse Bergen, which can be a reflection of the number of fingerprints in this zoo, since it had the highest number of samples, namely 15, among these 6 zoos.

The quantification of Clostridium cluster XIVa revealed that this cluster was present in all the fecal samples with the range of 3.04E+06-1.3E+11 CFU/g, except for animal NL14. This animal suffered from renal disease, which might have led to a decline in the quantity of Clostridium cluster XIVa (Van den Abbeele et al., 2010; Niwa, 2010).

In the future, more fecal samples should be collected and analyzed by DGGE to confirm the members of core microbiota and to assess the other members of the core microbiota. Different sorts of sequencing can be performed to determine the members of gut microbiota of captive cheetahs if necessary. Moreover, bands belonging to potentially discriminating band-classes can be sequenced to identify the unknown bacterial genera and species in the fingerprint profiles. Since multiple taxa can occupy the same position in the DGGE gel, as it is one of the shortcomings of DGGE, more zoomed DGGE can be performed to bring the members of gut microbiota of cheetahs to light.

As a general conclusion, this study has shown that Clostridium cluster XIVa, XI and I might be members of the core microbiota in these 36 captive cheetahs. Moreover, no zoo specific or diet type specific clustering was visualized in the DGGE profiles, which may indicate that the microbiota is affected by a combination of factors. Clostridium cluster XIVa was quantified in all the fecal samples except for an animal suffering from renal disease.
Materials and Methods

CHAPTER VII. MATERIALS AND METHODS

VII.1. FECAL SAMPLE COLLECTION

In this project, 55 fecal samples were collected from 36 adult cheetahs housed at 6 EAZA zoos in 4 European countries. The information about the housing, diet and background of the animals are summarized in (Table 1). Fecal samples were immediately collected upon defecation into sterile plastic bags or tubes, transported on dry ice and stored at -80°C until further analysis.

VII.2. HOMOGENIZATION OF FECAL SAMPLES

Since fecal samples from cheetahs are heterogeneous, homogenization is required prior to further analysis. This step is carried out in a biohazard.

In this procedure, 25 grams of each fecal sample was placed in a stomacher bag followed by homogenization in 225 ml bacteriological pepton (L37; Oxoid, Basingstoke, United Kingdom). The homogenate was then filtered on a Buchner funnel to remove the large undigested particles from the diet such as tendons and bones. Afterwards, the homogenate was centrifuged followed by removing the supernatant and adding 1x TE buffer to the pellet. In the last step the final homogenate was divided into 7 aliquots, which were stored at -80°C (See IX.2. page 64).

VII.3. DNA EXTRACTION

Total bacterial DNA was extracted from the fecal samples by using a slightly modified version (Vanhouette et al, 2004) of the protocol of Pitcher and coworkers (Pitcher et al, 1989). In this protocol, enzymatic and chemical lysis are used to recover DNA from the bacterial cells (See IX.3. page 64).

VII.4. DNA QUANTITY AND QUALITY CHECK

The DNA size and integrity were determined using 1% agarose electrophoresis gels stained with SYBR Safe. Additionally, DNA concentration and purity were assessed by spectrophotometric measurements at 234, 260, and 280 nm (See IX.4. page 65).

VII.4.1. AGAROSE GEL ELECTROPHORESIS

Agarose is a porous linear polysaccharide of beta-galactose with hydrogen bonds that break while heating.

In order to perform agarose gel electrophoresis, DNA samples with loading dye were loaded on 1% agarose gel located in a Tris-Borate-EDTA (TBE) buffer tank. After electrophoresis, the gel was stained in a SYBR Safe bath followed by visualization under blue light.
VII.4.2. OPTICAL DENSITY MEASUREMENT BY SPECTROPHOTOMETER

In order to determine the quantity and quality of the extracted DNA, spectrophotometric measurement at 260nm, the maximum absorption of light by DNA, 234nm, the minimum absorption of light by DNA and 280nm, the maximum absorption of light by proteins was performed. The ratio of OD260/OD280, a measure for DNA purity, should be between 1.8 and 2.2, while OD234/OD260, which can indicate protein contamination, should be between 0.5 and 0.8.

VII.5. V3-16S RRNA PCR

Prior to DGGE, amplification of small fragments of DNA is required. The V3 region of the 16S rRNA was targeted with universal bacterial primers F+GC/F357 and R518. The forward primer contained a GC clamp to prevent the amplicons from complete denaturation by the gradient (Temmerman et al, 2003; Yu & Morrison, 2004) (Table 4). The final volume of each PCR mixture was 50ul. V3-16S rRNA PCR results in the production of amplicons 194 bp in length (Vanhoutte et al, 2004) (See IX.5. page 65).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Target</th>
<th>Primer Sequence</th>
<th>Product size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>F+GC-F357</td>
<td>V3-16S rRNA</td>
<td>5’-CGCCCCGCGCAGCGGCGGGGC</td>
<td>194 bp</td>
<td>(Temmerman et al, 2003; Yu &amp; Morrison, 2004)</td>
</tr>
<tr>
<td>R518</td>
<td>V3-16S rRNA</td>
<td>5’-ATTACCGCGGTGTGCTGCTG-3’</td>
<td></td>
<td>(Yu &amp; Morrison, 2004)</td>
</tr>
</tbody>
</table>

Table 4. The primers used for V3-16S rRNA PCR prior to DGGE. The forward primer, containing a GC clamp, and the reverse primer target the V3 region of 16S rRNA gene in the bacteria. The generated amplicons are 194 bp in size.

PCR consists of 3 major steps namely denaturation, annealing and elongation. In the first step double stranded DNA denatures by the high temperature (95°C). During annealing, the forward and reverse primers bind to the conserved regions alongside the V3 region of the 16S rRNA gene. Afterwards, the extension or elongation is carried out by Taq polymerase that uses the dNTPs to build double stranded DNA, namely the V3 amplicons. The PCR program used for V3-16S rRNA PCR is depicted in (Table 5). The size of the amplicons was checked on 1% agarose gel electrophoresis.

<table>
<thead>
<tr>
<th>N°</th>
<th>Primer set</th>
<th>Target</th>
<th>Temperature program</th>
<th>Reference</th>
</tr>
</thead>
</table>
| 15 | F+GC-F357/ R518 | V3-16S rRNA | ❖ 1x 95°C (1min)  
❖ 30x 95°C (30s)-55°C (45s)-72°C (1min)  
❖ 1x 72°C (7min)  
❖ 4°C | (Huys et al, 2008) |

Table 5. The PCR program used for V3-16S rRNA PCR.
Materials and Methods

VII.6. DGGE ANALYSIS AND GEL PROCESSING

The V3-16S rRNA amplicons were analyzed by DGGE fingerprinting using 35 to 70% denaturing polyacrylamide gels. Urea and formamide are the denaturing agents added to polyacrylamide to obtain a denaturing gradient on the gel. These components cause the amplicons to denature, based on their GC contents, while moving through the polyacrylamide gel during electrophoresis (Muyzer & Smalla, 1998).

Afterwards, the DGGE gels were stained in 1x SYBR Gold nucleic acid gel stain TAE buffer (catalog no. S-11494, Molecular Probes), and UV light was used for visualization. Digital images were generated from the DGGE profiles in order to perform data analysis (See IX.6. page 66).

DGGE band extraction: Bands belonging to potentially discriminating band-classes were excised from the DGGE gel, eluted in 40ul of 1x TE buffer and heated for 10 min at 65°C. Subsequently these DNA solutions were re-amplified using the same V3-16S rRNA primers as those of the community PCR.

Sequencing of V3-16S rRNA amplicons: Bands were sequenced using an ABI PRISM 3130x1 Genetic Analyzer (Applied Biosystems, Foster city, California USA). Sequences were obtained using both forward (F357 without GC clamp) and reverse (R518) primers. Sequence assemblies were achieved with Bionumerics v7.1 (Applied maths). Taxonomic classification was obtained by comparison against the NCBI database.

VII.7. DATA ANALYSIS

Similarities between DGGE fingerprint profiles of fecal samples were determined using the Pearson and Dice correlation coefficients and the unweighted pair group method with arithmetic mean (UPGMA) in BioNumerics version 7 (Applied Maths, St.-Martens-Latem, Belgium). Boxplots were generated in Statistical Package for the Social Sciences (SPSS) version 22, to compare the variability of pairwise similarities between all 55 fecal samples and between fecal samples per zoo, per diet type and per animal.

The band richness of each DGGE profile, which is the total number of bands per fingerprint, was determined using BioNumerics, followed by generating boxplots for band richness per zoo in SPSS version 22.

The fingerprints were then compared with the clone library, previously obtained from the fecal samples of 2 captive cheetahs in Belgium. The clone library consisted of 16S rRNA sequences of clones. Groups of sequences with ≤ 3% sequence divergence (≥ 97% similarity) were defined as an operational taxonomic unit (OTU) or phylotype. Representative clones per OTU were loaded on DGGE fingerprints and these bands were correlated with unknown bands in the fingerprints of the 55 samples.
Materials and Methods

VII.8. QUANTITATIVE PCR

VII.8.1. CALIBRATION OF A STANDARD CURVE

*Clostridium symbiosum* (R25411), which is a member of *Clostridium* cluster XIVa, freeze-dried in an ampulla, was used from the R collection of the laboratory of microbiology to construct a qPCR standard curve for *Clostridium* cluster XIVa. The ampulla was broken in a nutrient broth to awake the cells. From the broth, droplets were poured on a plate with reinforced clostridial agar medium and incubated anaerobically overnight at 37 °C. The cells were cultivated for several generations in the anaerobic chamber. Afterwards, the cells were harvested from massive end plates to make a bacterial suspension. A serial dilution was made from the main suspension until 10⁻⁸, followed by pouring 50µl of each dilution on M37 plates in triplicates (Figure 23). Consequently, plates were incubated overnight at 37 °C. The whole procedure was performed in the anaerobic chamber (See IX.8. page 67).

![Figure 23. Preparation of serial dilution to construct the standard curve for quantitative PCR followed by plating the dilutions to calculate CFU/ml. A serial dilution was made from Clostridium symbiosum (R25411), a representative of Clostridium cluster XIVa.](image)

From the countable triplicate plates (between 25-250 colonies), the mean of colonies were calculated as well as the CFU/ml by multiplying the dilution in the colonies mean in 20. From the initial bacterial suspension, DNA was extracted, according to the DNA extraction protocol for Gram positive bacteria (Pitcher *et al*, 1989). Quantitative PCR was then performed on the resulting DNA in order to build the standard curve for *Clostridium* cluster XIVa to enable the quantification of *Clostridium* cluster XIVa in the samples.
Materials and Methods

VII.8.2. QUANTIFYING CLOSTRIDIUM CLUSTER XIVa IN THE FECAL SAMPLES

Quantitative PCR amplification and detection were performed for quantification of Clostridium cluster XIVa, using the Roche Light Cycler 480 machine. The total volume of each PCR reaction was 20µl. Parts of V3 and V5 regions of the 16S rRNA gene of Clostridium cluster XIVa, as well as the whole V4 region were targeted by group specific primers (Table 6). The generated amplicons were 429bp in size.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Target</th>
<th>Primer Sequence</th>
<th>Product size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CcocRT-F</td>
<td>V3,4,5-16S rRNA</td>
<td>5'-CGGTACCTGACTAAGAAGC-3'</td>
<td>429 bp</td>
<td>(Rinttilä et al, 2004)</td>
</tr>
<tr>
<td>CcocRT-R</td>
<td>V3,4,5-16S rRNA</td>
<td>5'-AGTTT(C/T)ATTCTTGGAACG-3'</td>
<td></td>
<td>(Yu &amp; Morrison, 2004)</td>
</tr>
</tbody>
</table>

Table 6. The primers used for qPCR of Clostridium cluster XIVa. The forward and reverse primers target the V3, V4 and V5 regions of 16S rRNA gene in the bacteria. The generated amplicons are 429 bp in size.

The program used for amplification of Clostridium cluster XIVa consists of 4 major steps (Table 7).

<table>
<thead>
<tr>
<th>Program</th>
<th>Cycles</th>
<th>Temperature (°C)</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-incubation</td>
<td>1</td>
<td>95</td>
<td>10 m</td>
</tr>
<tr>
<td>Amplification</td>
<td>40</td>
<td>95</td>
<td>15 s</td>
</tr>
<tr>
<td></td>
<td></td>
<td>55</td>
<td>20 s</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72</td>
<td>30 s</td>
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<td>80</td>
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<td>Melting curve</td>
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<td>95</td>
<td>5 s</td>
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<td>65</td>
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<td></td>
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<td>97</td>
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<tr>
<td>Cooling</td>
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<td>10 s</td>
</tr>
</tbody>
</table>

Table 7. The quantitative PCR program for targeting Clostridium cluster XIVa.
References

CHAPTER VIII. REFERENCES


References


References


54
References


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References


Figure 24. The original DGGE gels. Pictures were taken from the DGGE gels to perform normalization in BioNumerics.
Addendum

**IX.1. FECAL SAMPLES COLLECTION**

1. The fresh fecal sample was collected with a sterile spoon and it was then placed in a sterile container.
2. The fecal sample was then placed on the ice and transferred to the laboratory of Microbiology.

**IX.2. HOMOGENIZATION OF THE FECAL SAMPLES**

1. The fecal samples were taken out of the baggage in the biohazards. The entire sample was placed in a sterile stomacher bag and mixed manually for further homogenization.
2. 25 grams of the fecal sample was measured and put in a sterile stomacher bag.
3. 225 ml of 1g/l bacteriological pepton (LP0037; Oxoid, Basingstoke, United Kingdom) was added to 25 grams of the fecal sample.
4. The bag was placed in the stomacher machine at 230spm for 30 seconds to blend thoroughly.
5. The homogenate was filtered on a Buchner funnel and the large undigested particles on the filter were thrown away.
6. Tubes were filled with 45 ml of the homogenate.
7. The homogenate was centrifuged for 10 minutes at 1000rpm at 15°C.
8. The supernatant was removed.
9. The pellet was resuspended in 5 ml of 1× TE (1mM EDTA, 10mM Tris-HCl) buffer.
10. 7 aliquots, each containing 1 ml, from each sample were prepared.

**IX.3. DNA EXTRACTION**

1. 1 ml of the homogenate in an Eppendorf tube was centrifuged for 10 minutes at 13000rpm.
2. The supernatant was removed and 1 ml 1x TE buffer was added to the pellet and centrifuged for 5 minutes.
3. After removing the supernatant, the pellet was resuspeneded in 150µl of the enzyme mix. The enzyme mix consists of 5 mg lysozyme powder, 40µl of mutanolysine and 110µl 1x TE buffer.
4. The tubes were incubated at 37°C water bath for 40 minutes.
5. 500µl GES (600g/L guanidiumthiocyanate, 200 ml/l 0.5 EDTA, 10g/l sarkosyl) reagent was added to the mixture and the tubes were mixed gently. Then they were put on ice for 10 minutes.
6. 250µl of NH₄Ac (578.1 g/l NH₄OAc) was added to the mixture. The tubes were set on ice for 10 minutes.
7. Afterwards, 500µl chlorpform/iso-amylalcohol (192 ml chlorpform, 8 ml iso-amylalcohol) was added to the mixture followed by thorough homogenization. The tubes were centrifuged for 20 minutes at 13000rpm.
8. The maximum volume from the top layer was transferred to a new Eppendorf tube to which 300µl chlorpform/iso-amylalcohol was added. Again it was shaken for homogenization followed by centrifugation for 20 minutes at 13000 rpm.
9. From the upper phase, 700μl was transferred to a new Eppendorf tube and ice cold isopropanol (0.54 of their volumes), was added to them followed by careful homogenization and centrifugation for 20 minutes at 13000 rpm.
10. The supernatant was removed and the pellet was washed 2 times with 150μl 70% ethanol. The pellet was air dried and dissolved in 150μl TE 1x buffer overnight at 4°C.
11. After two days, 7.5μl RNase (2mg/ml) was added to the dilution and incubated at 37°C for 90 minutes.

IX.4. DNA QUANTITY AND QUALITY CHECK

The DNA quantity and quality check was performed through optimal density by spectrophotometer and also through agarose gel electrophoresis.

IX.4.1. AGAROSE GEL ELECTROPHORESIS

1. From the DNA sample, 5μl was mixed with 2μl of the loading dye (4g sucrose, 25mg bromophenol blue, 6 ml TE).
2. The solidified 1% agarose gel (BIOzym) was placed in TBE buffer tank.
3. The DNA samples were loaded on the gel, located in the tank, as well as the smart ladder (Eurogentec).
4. Electrophoresis was run for 45 minutes at 75v.
5. The gel was stained in midori green bath for 30-45 minutes.

IX.4.2. OPTICAL DENSITY MEASUREMENT BY SPECTROPHOTOMETER

1. From the DNA sample, 5μl was mixed and vortexed slowly with 95μl of 1x TE buffer to make a 1/20 dilution.
2. Then they were transferred to Greiner 96 micro titer well plate. The first well was filled with 100μl of TE buffer.
3. The cuvet was filled with 1 ml of 1x TE buffer and placed in the spectrophotometer.
4. The quantity of the DNA samples in the plate was measured by the spectrophotometer at 260nm, 234nm and 280nm.

IX.5. V3-16S RRNA PCR

Materials:

- 33.75μl MilliQ water
- 6μl AmpliTaq PCR buffer (Applied Biosystems)
- 2.5μl dNTPs (Applied Biosystems)
- 2.5μl bovine serum albumin
- 2μl forward primer with GC clamp (Sigma)
- 2μl reverse primer (Sigma)
- 0.25μl heat stable taq polymerase (Applied Biosystems)
Addendum

Method:

1. A master mix was made by combining the PCR components.
2. From the master mix, 49μl was poured in each PCR tube on ice.
3. Then 1μl of each DNA sample, in a 1/10 dilution, was added to the PCR mix.
4. The PCR tubes were put in the thermo cycler.
5. After the run was over, the tubes were shortly centrifuged.
6. To each PCR tube, 15μl loading dye was added.
7. Agarose gel electrophoresis was performed for 45 minutes at 75v.

IX.6. DGGE

1. The buffer tank was filled with 7 liters of 2% TAE buffer (242g Tris-base, 57.1 ml Glacial acetic acid, 18.61g Na₂ EDTA).
2. The 100% acrylamide container was put on the stirrer for 2h to decrystallize.
3. The chambers were cleaned by using milliQ water.
4. Preparation of plates: The spacers and the plates were cleaned with ethanol and rinsed. Afterwards, the silicon grease was smeread on the back and front of the long edge of the spacers and they were put on the longer plate facing outside. The smaller plate was flipped on the bigger one. The clamps were fixed on the right and left sides of both plates. Consequently they were placed in the tray to further align it by a standard cupboard. This step was also visually checked and grease was smeread under the spacers. Then they were placed in the tray with a piece of parafilm underneath the plates.
5. Casting: Low gradient polyacrylamide gel was made by adding 7.8 ml 0% denaturing agent, 4.2 ml 100% denaturing agent, 100μl APS 10% and 8μl TEMED. The high gradient polyacrylamide gel was made by adding 3.6 ml 0% denaturing agent, 8.4 ml 100% denaturing agent, 100μl APS 10% and 8μl TEMED. Afterwards, the low percentage solution was poured in the left chamber and the faucet was opened and closed immediately to make sure the bridge is filled with the low concentration denaturing agent solution. If some low concentration leaks to the right chamber, it should be taken out and poured in the left chamber with a micropipette. Then the high concentration gradient agent solution was poured in the right chamber while checking the level equality in both chambers, followed by putting a magnet in it. The stirrer was turned on between 2 and 3. The pump and the chamber’s faucet were turned on simultaneously. The needle of the pump tube was put in between the two plates to pour the gradient gel in between the two plates. After the gel reached a certain level, buthanol was added to the top of the gel and the gel was left to solidify for 2 hours. The tank was set up on 60 degrees and the pump and heater were turned on.
6. After 2 hours, the buthanol on top of the gel was poured out followed by cleaning it twice with milliQ water. Absorbing paper was used to rinse between the plates and an appropriate comb was put between the plates with a 5mm distance from top of the gel. The stacking gel, which contains 5 ml 0% denaturing agent solution, 50μl APS 10% and 5μl TEMED, was poured on the comb an it was left to solidify for 30 minutes.
Addendum

7. After 30 minutes, the comb was removed and the wells were washed twice with hot buffer with avoiding making bubbles. Then they were put on the holder with the small plate facing inside. To make sure that the plates are tightly fixed on the holder, some hot buffer was poured between the plates and on the chamber of the holder.

8. Then 25μl of each sample was loaded with a sharp tip, from right to left of the gel. The holder with the plates was put in the buffer tank. The level of the buffer in the tank should not be higher than maximum when the holder is placed inside. After that the lid of the tank was closed and the machine was set on 990 minutes on 70 volt.

9. The day after the tank was turned off and the clamps were unfixed. The plates were transferred to the ethidium bromide room. The plates were separated and the gel was marked to make sure of the order of the samples. In order to stain the gel, it was put in the SYBR gold bath for 30-45 minutes.

10. The gel was slowly taken out and placed on the UV machine to take digital photos.

IX.7. BAND EXTRACTION AND SEQUENCING

1. While taking an image from the DGGE under the UV light, a piece of the desired band was taken out by using a filter tip.
2. The band was put in 40μl of 1x TE buffer.
3. V3-16S rRNA PCR was performed and checked by 1% agarose gel electrophoresis.
4. The amplicons were poured on Macherey-Nagel 96 plates and it was filtered.
5. From the MilliQ water, 100μl was added to the amplicons in the plates and vacuumed.
6. From the MilliQ water, 100μl was added to the top of the filters.
7. The water and the amplicons were transferred to new Eppendorfs.
8. A master mix was prepared by combining 1.857μl MilliQ water, 1.857μl sequencing buffer, 0.286μl BigDye v3.1, 3μl forward primer (F357) and 3μl of the DNA sample.
9. A master mix was prepared by combining 1.857μl MilliQ water, 1.857μl sequencing buffer, 0.286μl BigDye v3.1, 3μl reverse primer (R518) and 3μl of the DNA sample.
10. The PCR tubes were set in the thermo cycler machine.
11. After the run was over, the amplicons were sent for sequencing.

IX.8. Q-PCR

IX.8.1. BUILDING A STANDARD CURVE

1. The content of a Clostridium symbiosum ampulla (R-25411) was poured in a nutrient broth in the anaerobic chamber.
2. From the broth, some droplets were poured on M37 plate and incubated in the anaerobic chamber at 37 °C overnight.
3. Several generations were prepared for the bacteria by the streaking technique as well as the massive end.
4. The bacterial cells were harvested from massive end plates by öse and diluted in 4 ml of bacteriological pepton (L37; Oxoid, Basingstoke, United Kingdom).
5. A serial dilution was made from the main dilution till 10⁻⁸.
Addendum

6. The tubes were thoroughly vortexed. From each tube, 50μl was poured on a M37 plate in triplicates.
7. The plates were incubated at 37°C overnight in the anaerobic chamber.
8. The countable plates were counted and CFU/ml was calculated by multiplying the mean number of colonies in 20 in the dilution.
9. The DNA was extracted from the 10^5 dilution according to Pitcher method (Pitcher et al, 1989) for gram positive bacteria.
10. V3-16S rRNA PCR was performed.
11. Quantitative PCR was performed as described in IX.8.2.

**IX.8.2. QUANTIFICATION OF CLOSTRIDIUM CLUSTER XIVa**

1. The PCR mix was prepared by mixing 10μl 2xSensi mix SYBR NO-ROX, 6.4μl MilliQ water, 0.3μl Ccoc-RT-F, 0.3μl Ccoc-RT-R and 3μl of the DNA sample (1/10 dilution). The total volume was 20μl per well.
2. The 96 plate was tightly sealed and it was centrifuged for 1 min at 0.7 rpm.
3. The 96 plate was placed in the Roche Light Cycler 480 machine and the protocol for quantifying Clostridium cluster XIVa was chosen.
Figure 25. Neighbor-joining phylogenetic tree showing the nearest phylogenetic related type strains for recovered OTUs from two 16S rRNA clone libraries from captive cheetah feces. Bootstrap values, expressed as percentages of 1000 replications, above 50% are given at branching points. The scale bar shows 5 nucleotide substitutions per 100 nucleotides. Number of clones in parentheses follows label of either common OTUs (framed), OTUs solely from CL-B1 (green) or CL-B2 (purple). Figure obtained from: http://www.biomedcentral.com/1471-2180/14/43/abstract (Becker et al, 2014)