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DIVERSITY AND DYNAMICS OF GUT MICROBIOTA IN CAPTIVE CHEETAHS (*ACINONYX JUBATUS*):

A BASELINE FOR DIETARY INTERVENTIONS IN A STRICT CARNIVORE WITH VULNERABLE STATUS

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Dissertation submitted in fulfilment of the requirements for the degree of
Doctor (Ph.D.) of Veterinary Sciences

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LM-UGent

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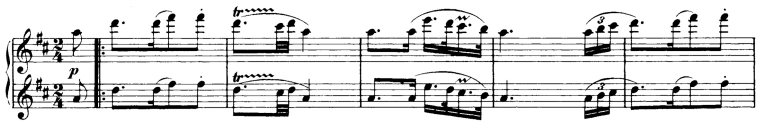

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ABBREVIATIONS

ANI	average nucleotide identity
ANOSIM	analysis of similarity
ANOVA	analysis of variance
ARISA	automated ribosomal intergenic spacer analysis
BB	Safaripark Beekse Bergen (The Netherlands)
BCFAs	branched-chain fatty acids
Bcl	band-class
BLAST	basic local alignment search tool
BO	Boras Djürpark (Sweden)
CCD	charged-coupled device
CCF	Cheetah Conservation Fund (Namibia)
$CD4^+T$	cluster of differentiation 4 - T helper cells
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
CO	Colchester Zoo (United Kingdom)
Ct	cycle threshold
CV-ANOVA	cross-validated analysis of variance
DACC	Data Analysis and Coordination Center
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
EAZA	European Association of Zoos and Aquaria
EB	Reepark Ebeltoft Safari (Denmark)
EDTA	ethylenediaminetetraacetic acid
EEP	European Endangered Species Programme
ER	Zoo Parc Erfurt (Germany)
ESVCN	European Society of Veterinary & Comparative Nutrition
FA	fatty acids
FDR	false discovery rate
FFA	free fatty acids
FISH	fluorescent *in situ* hybridization
GA	Gaia Zoo (The Netherlands)
GC	goblet cell
GIT	gastrointestinal tract
GLP-1	glucagon-like petide-1
HMP	Human Microbiome Project
IBD	inflammatory bowel disease
IgA	immunoglobulin A
IHMS	International Human Microbiome Standards
IS	intergenic spacer
ITIS	Integrated Taxonomic Information System
IUCN	International Union for Conservation of Nature
KEGG	Kyoto Encyclopedia of Genes and Genomes
m/z	molecular weight-to-charge

MALDI-TOF MS	matrix assisted laser desorption/ionisation time-of-flight mass spectrometry
MAMPS	microorganism-associated molecular patterns
MBQC	Microbiome Quality Control Project
MGB	microbiota-gut-brain axis
MO	Zoo de Montpellier (France)
MS	mass spectrometry
M-SHIME	Mucosal Simulator of the Human Intestinal Microbial Ecosystem
MU	Allwetterzoo Münster (Germany)
NGS	next-generation sequencing
OPLS-DA	orthogonal partial least squares with discriminant analysis
OTUs	operational taxonomic units
OV	Zoo Parc Overloon (The Netherlands)
PCA	principal component analysis
PCoA	principal coordinates analysis
PCR	polymerase chain reaction
PL	Planckendael (Belgium)
PYY	peptide tyrosine tyrosine
qPCR	quantitative polymerase chain reaction
RCM	reinforced clostridial medium
RDP	The Ribosomal Database Project
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SCFA	short-chain fatty acids
SDE	sequence-dependent electrophoresis
SHIME	Simulator of Human Intestinal Microbial Ecosystem
SI	Réserve Africaine de Sigean (France)
spp.	species
SSP	Species Survival Plan
TG	triglycerides
TGGE	temperature gradient gel electrophoresis
TRF	terminal restriction fragment
T-RFLP	terminal restriction fragment length polymorphism
TTGE	temporal temperature gradient gel electrophoresis
UPGMA	unweighted pair group method with arithmetic mean
WI	Wilhelma Zoologisch-Botanischer Garten Stuttgart (Germany)
ZZ	Zagreb Zoo (Croatia)

PART I | PREAMBLE

The research leading to this dissertation results from an interdisciplinary synergy between two laboratories at Ghent University. In 2010, the Laboratory of Animal Nutrition (Faculty of Veterinary Medicine) and the Laboratory of Microbiology (Faculty of Sciences) initiated a collaborative project to bridge the knowledge gap between dietary variation and intestinal metabolism in captive cheetahs by achieving a better understanding of the diversity and dynamics of their intestinal microbial ecosystem. The work was performed at the Laboratory of Microbiology (LM-UGent) and financially supported by the Special Research Fund of Ghent University (Belgium) (BOF/10/2JO/254 and BOF11/24J/122), by the Morris Animal Foundation (USA) (Grant D12ZO-404) and by an ESVCN/WALTHAM Research Grant. None of the funders had a role in study design, data collection and analysis, decision to publish or preparation of the dissertation.

This project also relies on the international collaboration between zoos under the umbrella of the European Association of Zoos and Aquaria (EAZA). The mission of EAZA is to facilitate cooperation within the European zoo and aquarium community for education, research and conservation. Zoos and aquaria have an important role to play in protecting nature and wildlife. From fundamental research to field observations, they are a crucial partner to improve our understanding of all aspects of animal biology. Without the support and cooperation of the European zoo community, this research project would not have been feasible.

The dissertation is organized as follows. **Part II** contains three chapters in which a comprehensive literature overview is given on the diverse and complex microbial community residing in mammalian guts, with a particular emphasis on the mammalian clade Carnivora. **Chapter 1** elaborates on the origin of the cheetah and current threats and challenges for its survival on earth. **Chapter 2** describes the diversity of the gastrointestinal microbiota and beneficial microbiota-associated functions in mammalian host physiology. Next, an overview of state-of-the-art techniques to analyze the structure and functions of this microbial ecosystem is presented, with a special focus on *in vitro* models. Finally, **Chapter 3** seeks to ascertain from currently available literature the gut microbiota signature associated with carnivorans, notably with feline species.
In **Part III**, the main objectives of the thesis are described in relation to the experimental work. The results of that work are presented in **Part IV** which is arranged into five chapters (**Chapters 4-8**), covering the various research questions defined in this project. **Part V** concludes the dissertation with a reflection on the work performed and a message to take home (**Part VI**).

PART II | LITERATURE STUDY

1 MEET THE CHEETAH

1.1. Carnivorans and carnivores disentangled

With over 270 extant species, the order Carnivora is one of the most diverse mammalian clades. Beyond the quirky cats and loyal dogs residing in our homes, this clade encompasses many of the world's top predators (e.g. lion, leopard, tiger, cheetah, hyaena, wolf) and iconic wild animals (e.g. giant panda, polar bear, grizzly bear). Evolutionary, Carnivora are divided into two evolutionary branches, *i.e.* Feliformia (including civets, genets, linsangs, mongooses, hyaenas and cats) and Caniformia (including the red panda, dogs, wolverines, bears, walruses, seals, skunks, raccoons, otters, badgers and weasels) [1]. Carnivorans (*i.e.* members of the order Carnivora) inhabit all of the world's oceans and five of the continents, with only Oceania and Antarctica lacking native terrestrial species. Accordingly, they display a broad range in styles of locomotion, including fossorial, arboreal, cursorial and aquatic species. Although the order is named after its meat-eating members (Latin, *carno*: flesh + *vorare*: to devour), the dietary breadth of carnivorans extends from frugivorous to insectivorous taxa, omnivorous taxa as well as the strict carnivorous taxa, which are mostly associated with this clade. Such diversity of diet is paralleled by a diversity in craniodental morphology. The key anatomical characteristic is the dental modification called carnassials and refers to the blade-like upper fourth premolar and lower first molar, which shear against each other for enhanced meat-slicing ability. Differences in carnassial morphology can be highly informative concerning the diet and separate carnivorous, omnivorous and herbivorous taxa [2]. The term **'carnivore'** is thus an ecological classification describing any meat eater and should not be confused with the phylogenetic classification **'carnivoran'**.

1.2. Origin and evolution of the species

Cryptic footprints of historic episodes encountered by ancestral progenitors are nestled in the genome of every individual. The recent revolution in DNA sequencing was spearheaded by the Human Genome Project [3], and also the first cat genomes are now out of the bag [4,5]. These genomes were instrumental to clarify the murky history of the *Felidae*, a subject of debate ever since Fischer de Waldheim (1817) erected the family as part of the Feliformia (ITIS; www.itis.gov, accessed July 12th, 2015). Originating from a pantherlike predator that lived in Southeast Asia 10.8 million years ago, all 37 extant species of the family are now classified into eight clades. The great roaring cat lineage was the first to branch off and seven other lineages followed. The puma lineage including the genera *Puma* and *Acinonyx* split off 6.7 million years ago, and cheetahs (*Acinonyx jubatus*) are the only remaining living members of the *Acinonyx* genus, classified into four African and one Asian subspecies (**FIG 1.1**) [6,7].

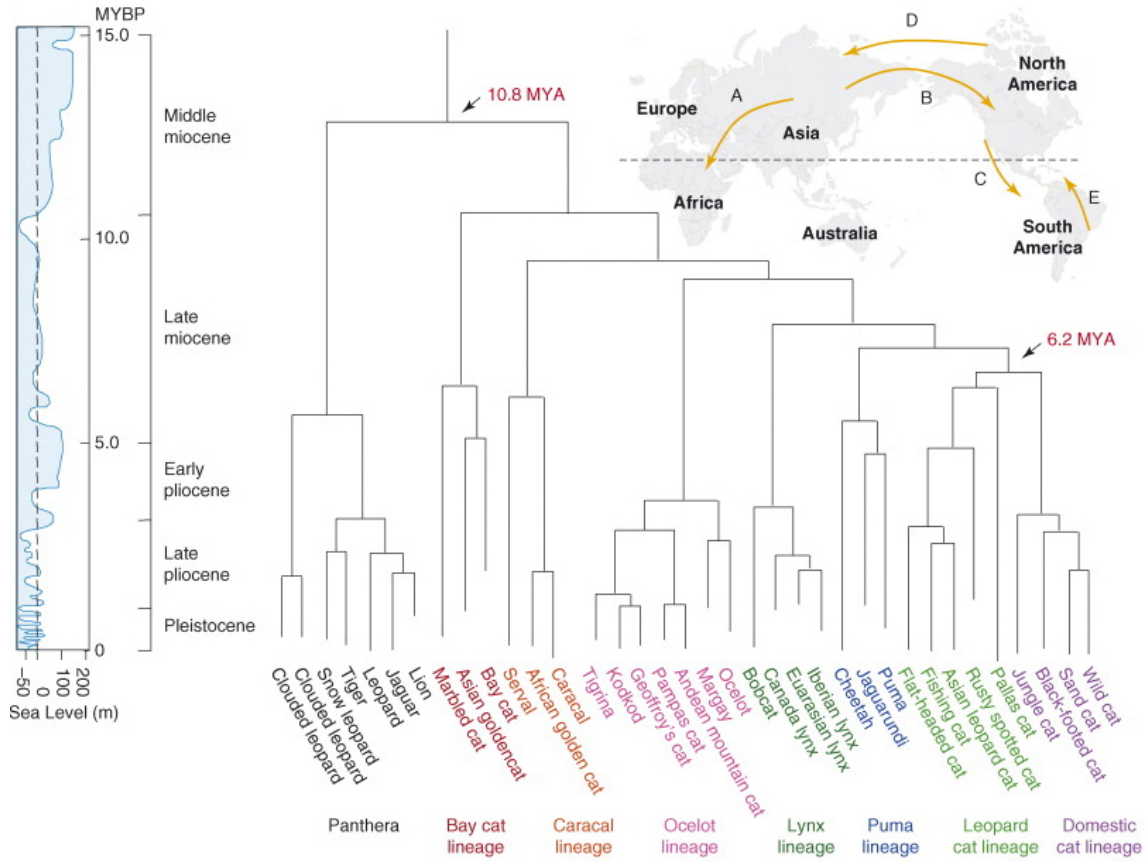

FIG 1.1 | Molecular phylogeny of *Felidae*. The branching hierarchy of the cat family as discerned from an analysis of 30 genes from members of each species. The time scale is imputed from several dates of fossils that define certain portions of the tree (arrows). The geological periods during the 11 million year interval of cat evolution are presented beside the time scale. The global sea levels relative to current sea level are shown in metres. Arrows A to E depict five imputed ancestral migration routes that were traversed by ancestors of modern cat species. (Figure reprinted from Trend in Genetics 24, State of cat genomics, O'Brien *et al.*, 2008, with permission from Elsevier) [6]

In general, the cheetah is regarded as a genetically depauperate species due to a demographic bottleneck that occurred approximately 12 000 years ago and a more recent anthropogenic-related bottleneck in the 20th century [8]. The low genetic variability resulting from subsequent inbreeding has been suspected as the cause of reduced sperm quality in males, high neonatal mortality, increased disease susceptibility, morphological abnormalities (e.g. kink in tail and focal palatine erosion) and an overall population decline [9–12]. However, some of these potential consequences of genetic homozygosity have been revisited. A recent study showed that low polymorphisms in genes of the major histocompatibility complex seemed not to influence the immunocompetence of cheetahs [13] and although low levels of heterozygosity persist, other bottlenecked populations (e.g. Scandinavian beavers and Northern elephant seals) exhibit even lower levels and yet have survived and even increased in numbers [14–17]. Furthermore, differentiation at both nuclear and mitochondrial levels support greater distinctiveness between the cheetah subspecies than originally thought [18]. The evidence that reduced genetic diversity affects cheetah population viability is so far equivocal but in light of the critically low numbers, it is vital to the survival of the world's cheetah population to preserve their genetic footprint.

1.3. World's fastest land mammal running its race for survival

In 2012, an 11-year old cheetah shattered the 100-m dash world record, clocking a time of 5.95 seconds, with an average speed of 61 km/h and a peak speed of 98 km/h. For comparison, it takes Usain Bolt still 9.58 seconds to reach that finish line. Cheetahs are the most unique and specialized members of the cat family and aerodynamically built for sprinting to a full speed of 110 km/h in seconds during diurnal hunting. Its slender body with flexible spine and small waist supports long limbs with semi-retractable claws and a deep chest cavity with a powerful heart. The enlarged nostrils and extensive sinuses allow greater amounts of air to enter the lungs, and its muscular tail is particularly long to provide extra balance [19]. As a price for its speed, the small head holds small teeth and weak jaws with a considerably lower bite force quotient [20]. Consequently, relative to other apex predators, cheetahs are non-aggressive animals, choosing flight versus fight and are often robbed of their hard-won kills and their litter. In the wild, cheetah cubs have even less than 10% chance of reaching sexual maturity (± 20 months) [19].

BOX I – Tears of the cheetah

When the world was first created, all the animals came down onto the savanna. The cheetah got used to the new world it was in and ran around. Soon, the cheetah grew lonely and went to look for more of her kind. The cheetah spotted some big cats in the distance and ran to them. They were lions. The cheetah told the male lion about how she was looking for her own kind and the lion told the cheetah that she was no lion. She was much too thin to be a lion and her claws were like a dog's. Feeling discouraged, the cheetah continued her search. She came across a pack of wild dogs and told them what the lion had said. They all howled with laughter. They said the cheetah was no dog because of her round head and ears and rough tongue. The cheetah ran away in fear. When she finally stopped, she laid down under a tree. The cheetah felt so sad about her rejections that she began to cry. A giraffe walked up quietly and asked the cheetah why she cried. The cheetah told the giraffe about what the lions and wild dogs had said and how she had cried so much that her beautiful face was now stained with black tear streaks. A bird flying by saw the cheetah and told her how he has travelled the land and she was the most splendid and unique of all the cats. From that day, the cheetah had its tears and chirped to show how proud it was to be the most unique of all the cats. (African legend)

The interspecies competition with other large carnivores is a major threat to the free-ranging cheetah, enforced by large anthropogenic habitat loss and fragmentation. Cheetahs need however large expanses of land to survive. Depletion of the wild ungulate prey base, replacement with livestock and concomitant persecution by farmers and hunters are also considered significant threats [21]. Consequently, cheetahs have disappeared in the past century from ± 76% of their historic habitat originally ranging from Southern and East Africa over the Middle East to Central Asia (**FIG 1.2**) [22].

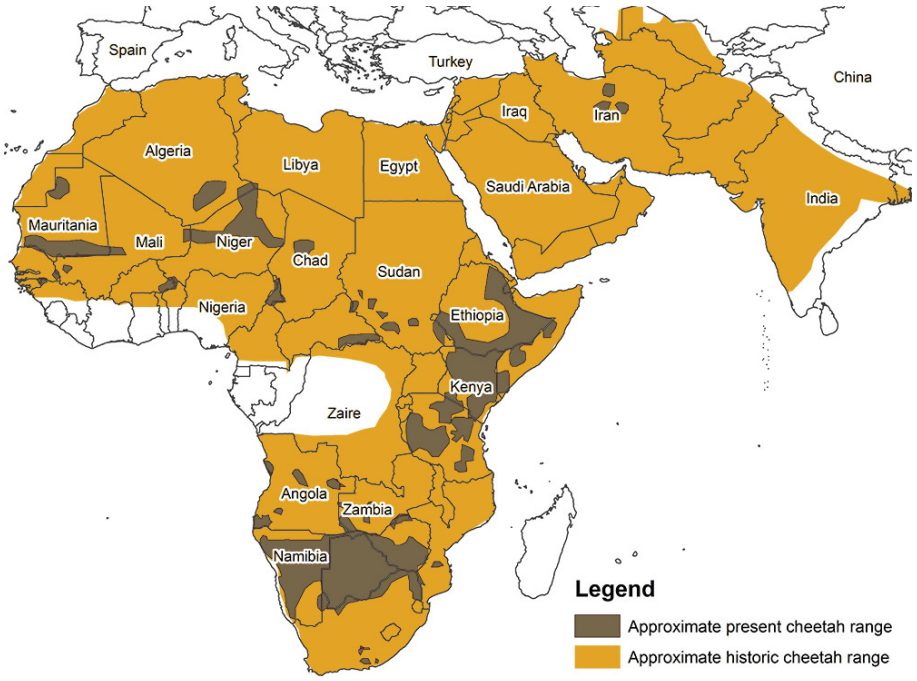

FIG 1.2 | Historical (anno 1900) and present (2010) habitat range of free-living cheetahs. (adapted with permission from CCF; http://cheetah.org/)

Cheetahs are nowadays listed on the IUCN Red List of threatened species as 'vulnerable' in sub-Sahara Africa and 'critically endangered' in Northwest Africa and Iran (IUCN; www.iucnredlist.org, accessed July 13th, 2015). Furthermore, they are protected under several major treaties and laws including CITES Appendix I which imposes prohibition of trade in live animals or products and restricted quota for hunting trophies [23]. Yet, the cheetah population lingers in fast decline, with on average 7 000 cheetahs left in the wild. The problem facing the cheetah is complex and multifaceted. To help this species endure, combined support from scientists, land owners, policy makers, educators and the public is required. Also the captive cheetah population, registered in September 2014 at 1745 animals [24], provides a truly invaluable resource for education and research. Many facilities housing cheetahs are involved in regionally and internationally managed breeding programmes (e.g. European Endangered Species Programme [EEP] and Species Survival Plan [SSP]) [25,26]. However, the breeding programmes of our world's zoos are not self-sustaining and thus one of the main goals is to manage the captive cheetah population without the need for wild-caught animals.

1.4. Challenges to *ex situ* conservation

1.4.1. Implications of captivity

The European captive cheetah population holds 436 registered animals housed at 104 facilities [24]. Even though populations in captivity are important for the cheetah's global persistence, they are a challenging species to manage in zoos and complicate husbandry practices. Historically, zoos have always experienced difficulties breeding cheetahs. Apart from the genetically derived teratospermia in male cheetahs [27], oestrus cyclicity is highly variable in captive females due to prolonged periods of ovarian inactivity [28], potentially related to socially induced reproductive suppression when housing female cheetahs in groups [29]. Another major impediment to successful captive propagation is the inability to identify or predict the occurrence of oestrus [30]. Unnatural husbandry conditions in terms of public exposure, number of care-givers, space, temperature and feed have also been attributed as potential causal factors [31–33].

In contrast to the free-ranging populations, a higher incidence of gastrointestinal and metabolic diseases impairs captive cheetah populations, in particular chronic gastritis, glomerulosclerosis, amyloidosis and veno-occlusive disease [32,34,35]. There is considerable concern that chronic stress, suggested by higher faecal corticoid concentrations and larger adrenal cortices [36], contributes to these major causes of mortality in captive cheetahs. Unusually severe gastritis affects at least 55% of the European population and is associated with multiple *Helicobacter* spp., though no cause-effect relationship has been established [34]. In contrast, free-ranging cheetahs develop only mild inflammatory lesions when infected with similar *Helicobacter* spp. [32]. This has triggered the hypothesis that a stress-related altered response to commensal bacteria might be present in the captive population [37]. In support of previous assumptions regarding the possible role of diet in the development of gastritis [38], also nutritional risk factors (e.g. increased crude protein intake) have recently been identified in a large scale cross-sectional epidemiological study [39]. Still, more detailed evaluation of nutrient composition as well as serum biochemistry is warranted to provide evidence of diet suitability for the maintenance of gastrointestinal health in captive cheetahs [39]. Other nutritional considerations include the skewed calcium to phosphorus ratio in food items such as muscle meat and liver. Insufficient supply of dietary calcium may contribute towards developmental deformity of the forelegs, enlarged joints, angular limb deformities and stunted growth in cubs [19,40]. Diet-induced hypervitaminosis A has also been reported as potential cause of skeletal malformations, reduced liver function, enteritis and conjunctivitis [41]. On the other hand, commercially prepared diets, balanced for essential nutrients, have been linked to a higher prevalence of liquid faeces and vomiting [39] and its soft structure has been shown to be detrimental to oral health and feeding behaviour [42]. But then again, feeding of raw meat diets has been associated with food-born diseases such as salmonellosis and clostridial disease [43]. The resulting enterocolitis should be differentiated from uncomplicated colitis commonly seen in adult captive cheetahs. Cheetahs with uncomplicated colitis generally have abnormal stool with varying amounts of

mucus and fresh blood but, otherwise, appear normal. Reported etiologic agents are *Clostridium perfringens* (enterotoxicosis), *Plesiomonas shigelloides*, *Salmonella* spp. (salmonellosis) and feline enteric coronavirus, although idiopathic colitis is frequently diagnosed as well [41,44,45]. In addition, the resulting intestinal inflammatory reactions tend to be more severe in captive cheetahs [41].

1.4.2. Considerations for captive cheetah population management

Whereas the cheetah's genetic makeup is beyond our control, we can adapt our animal management strategies to create conditions for optimal well-being. Ideally, the framework of a holistic *ex situ* conservation strategy comprises several pillars. Some of these are briefly outlined below but compiled in elaborate detail in the Husbandry Manual for the Cheetah [41]. In the frame of this thesis, emphasis is put on nutritional considerations.

Of primary importance for proper cheetah management is the ***basic facility design***, which includes preferably as large as possible outdoor enclosure(s) with flexibility to move cheetahs into different enclosures and quarantine areas. Visual contact with other large carnivores should be avoided whereas a wide view of the surrounding area including hoofed animals may provide visual and mental stimuli. Indoor enclosures require proper ventilation, temperature and hygiene control, and measures for safe handling and animal restraint. The latter can benefit the ***behaviour and stress management*** of the cheetahs. Taking the social organisation of this species into account in management strategies is known to beneficially affect animal behaviour. As such, males should be housed alone or in coalition, whereas females are preferably housed alone, especially when they are recommended for breeding [29]. Proper ***breeding management*** not only results from proper facility design and flexibility, but also from detailed knowledge of individual courtship behaviour [30]. Exercising cheetahs on a lure coursing system is another management tool promoting species-appropriate behaviour. Other ***enrichment strategies*** include logs and deadfall in the outside area to promote climbing, scratching, scent marking and to act as promontories for cheetahs to survey their home range. ***General health assessment*** of the cheetahs should include ectoparasite control, body condition scoring, collection of individuals' faeces for routine parasite check and endocrine research, and a vaccination and deworming schedule. Finally, of the utmost importance are ***optimal nutritional strategies*** since nutrition underpins the majority of health parameters.

Whereas the wild or natural diet of cheetahs is not necessarily the optimal one, it certainly is a reference point. The consumption of prey in the wild (e.g. antelopes, hares and wildebeests) not only includes muscle meat, but also small bones, cartilage, tendons, fur, skin and organs [19]. Valkenburgh estimated that in the wild, feeding on muscle made up 40-60% of the cheetah's diet, whereas feeding on skin and connective tissues comprised 17-24% and 12-27%, respectively [46]. Cheetahs are not observed to feed on large bones, but ± 8% of the diet can include small bone structures of prey such as skulls and ribs. From a large international survey conducted in 86 cheetah-holding facilities, raw meat feeding was reported as the most common practice [39]. Whereas carcass-feeding was mainly restricted to European and some

African facilities, North American facilities typically fed commercially prepared soft diets which mostly consist of vitamin- and mineral-supplemented frozen minced horse meat without bones, cartilage, organs, skin or connective tissues. The latter type of diets is thus readily available and formulated on a sound nutritional basis, though mostly derived from studies in the domestic cat. These diets provide appropriate protein-to-fat ratio of 2-4:1, calcium-to-phosphorus ratio of 1-2:1 and high nutrient digestibility [47] but fail to stimulate natural feeding behaviour and are detrimental to oral health [42]. Raw chunked meat diets are an alternative provided that they are sufficiently and in a correct way supplemented with various vitamins and mineral supplements [48]. Recent research advocates for increased incorporation of whole prey items since they do not only affect feeding behaviour and improve satiety but also provide different types of animal tissues which are likely to influence both host digestion and gut microbial fermentation [19,49,50]. The effect of non-digestible but potentially fermentable dietary components from plant origin (e.g. indigestible non-starch polysaccharides, pectins, oligosaccharides, lignin, [hemi]cellulose and gums) has been extensively studied in domestic cats through *in vitro* and *in vivo* studies [51]. Likewise, non-digestible tissues of whole prey (e.g. fur, cartilage, skin, bone) have been postulated to act as animal fibre in cheetahs. Bone mainly consists of 65% inorganic hydroxyappatite crystals and 35% organic matter which includes collagen (90%), mucopolysaccharides and non-collagenous proteins (e.g. osteocalcin) (10%). Cartilage is mainly made up of water, collagen (60% of dry matter), proteoglycans, phospholipids and glycoproteins. The key structural material of hair and skin is keratin, a fibrous structural protein. These compounds have been put to the test through an *in vitro* and *in vivo* study. Consumption of whole carcasses resulted in a tendency towards improved stool consistency and changed fermentation profile with decreased total branched-chain fatty acids and putrefactive compounds such as indole and phenol [49,52]. Additionally, feeding whole rabbits resulted in decreased faecal S100A12 protein concentrations, markers of intestinal inflammation [53].

So far it has not been possible to link observed fermentation patterns to specific bacterial shifts or adaptations in the intestinal ecosystem because the diversity and dynamics of the functional gut microbial members in the cheetah is unknown. Still, these studies have provided novel insights into a largely overlooked aspect of nutrition in exotic felids dealing with the potential of enzymatically indigestible dietary components to contribute to host health by acting upon gastric emptying, satiety, nutrient digestibility, motility, transit time and fermentation. The research questions drawn from this warrant further investigation and empirical testing as they are relevant to the ultimate goal of any managed feeding programme: proper diet formulation based on sound nutritional concepts and quality sources of dietary components, presented in ways to promote natural feeding behaviour.

2 GUT MICROBIOTA, THE INNER NATURE OF MAMMALS

2.1. A historical gut feeling

"I then most always saw, with great wonder, that in the said matter there were many very little living animalcules, very prettily a-moving" Antony van Leeuwenhoek wrote to the Royal Society of London on September 12th, 1683 [54]. The observations from scrapings of his own teeth made van Leeuwenhoek the first to study and describe bacteria, marking the beginning of microbiology. In subsequent centuries, the field expanded as many famous pioneers discovered the distribution of microorganisms on practically every square metre of the planet and their phenomenal impact on life. Although many free-living microorganisms are known to significantly influence diverse ecological processes, the microbial communities that leave us most astonished are those living in intimate partnership with other (higher) organisms (*i.e.* symbiosis). As David Relman surmised most eloquently: symbioses are the ultimate examples of success through collaboration and the powerful benefits of intimate relationships [55]. As partners in such a relationship, the fascination of humans for the collection of microscopic creatures that make their home in and on us, (*i.e.* **the human microbiota)**, and their genes (*i.e.* **the human microbiome**) spurred scientists to the development of molecular sequencing techniques. These allowed us to make observations far beyond van Leeuwenhoek's microscope and to discover trillions of tiny creatures living on our eyes and ears, skin and genitals. Different sets of micro-organisms including Archaea, fungi, viruses and bacteria inhabit different body sites, but the most complex and densely populated microbial communities so far documented are the bacterial assemblages residing in the magnificent mansion that is our gut. As one of the first major joint efforts to take a genetic census of a healthy microbiome, the Human Microbiome Project was launched in 2008 (www.hmpdacc.org) as well as the MetaHIT consortium (www.metahit.eu) which is financed in large part by the European Union. Other international initiatives followed, resulting in the fact that most of our current understanding of mammalian intestinal microbiota comes from studying the human gut microbial ecosystem. Soon, new microbiological frontiers extended beyond the human body as the same technologies allow us to explore the gut microbial communities associated with pets, livestock, wild animals and many more. This endeavour of microbial discovery holds the potential to redefine our understanding of animal physiology, mechanisms of animal and zoonotic diseases, animal health and care, habitat-associated host fitness and mammalian host-microbe co-evolution.

2.2. Microbial diversity in the mammalian gut

2.2.1. It all starts at birth

In mammals, early microbial colonization and diversification into a stable ecosystem take place during a critical early-life period of gut maturation and immune development. This process is necessary to establish host-microbe interactions in a carefully orchestrated manner and thus create optimal symbiosis. Any significant disturbance may hold future health consequences.

A newborn's gut was thought to be sterile, but the recent reporting of a placental microbiome and microbes in the meconium challenge this assumption [56]. Minutes after birth, the newborn's gut is colonized by microbial pioneers derived from the maternal microbiota (vaginal, faecal, milk, mouth, skin) and the environment during a process of natural selection, commonly referred to as ecological succession [57–59]. Not surprisingly, the mode of delivery (*i.e.* natural birth vs. caesarean section) plays a pivotal role in the microbial steering of these early colonization events. It has been shown that vaginally delivered babies acquired bacterial communities resembling the vaginal community of the mother, whereas pioneering microbiota of caesarean delivered babies resembled more skin communities [60]. This observation holds clinical importance as the presence or absence of intimate contact with maternal microbiota is thought to influence risk patterns of disease development (*i.e.* obesity, food allergies and atopic dermatitis) later in life [61]. In addition, numerous other factors profoundly influence early gut microbial assembly including geographic origin of birth [62], sanitary conditions [63], antibiotic treatments [59] and initial diet (breast vs. formula feeding) [64–66]. The first settlers in the human gut are facultative anaerobic bacteria such as *Enterococcus*, *Enterobacter*, *Streptococcus* and *Staphylococcus* spp. These early colonizers create an anaerobic environment and provide substrates (e.g. succinate and lactate) for successive colonization by obligatory bacterial anaerobes such as *Bifidobacterium*, *Bacteroides*, *Clostridium* and *Eubacterium* spp. [66,67]. This first dynamic variable stage of early succession is followed by another rapid and important shift when introducing solid foods and/or weaning. The early predominance of *Bifidobacterium* spp. shifts towards dominating levels of bacterial species belonging to the phyla Firmicutes (*Roseburia*, *Ruminococcus*, *Clostridium* and *Lactobacillus* spp.) and Bacteroidetes (*Bacteroides* spp.). Associated metabolic profiles evolve towards an increased production of propionate and butyrate resulting from carbohydrate breakdown and cross-feeding of early metabolites [68]. A gradual increase in complexity will finally constitute the adult-like microbiome which is generally established in humans around three years after birth, but still changing during childhood [69].

Compared to humans, studying microbial pioneering and associated influencing factors in the gastrointestinal tract (GIT) of other mammals is still in its infancy. Microbial succession in most animals is believed to undergo relatively similar patterns, although lactobacilli generally predominate compared to bifidobacteria in humans. Neonatal piglets harbour a consortium of bacteria holding different *Lactobacillus* spp., *Escherichia coli* and *Shigella flexneri*. After the weaning process, which is an abrupt,

early and critical transition from milk onto a solid diet in commercial animal production settings, lower levels of lactobacilli and emergence of clostridia and *E. coli* were observed [70–72]. In these intensive husbandry systems, the early-life environmental variation in terms of antibiotic treatment and stressful handlings has recently been shown to influence this process profoundly [73]. Likewise, differences between calf rearing systems (*i.e.* particular feeding regimes and age of weaning) have been reported to influence microbial community assembly in the calves' GIT. The relative abundance of *Bacteroides*, *Escherichia-Shigella* and *Lactobacillus* spp. before weaning shifts in favour of the *Oscillibacter* spp. and members of the *Prevotella* group which are influenced by a high-fibre diet [74,75]. Long before weaning or exposure to plant material, however, bovine rumen communities hold cellulolytic bacteria important to proper functioning of the adult rumen. The development of this ruminant-specific community is mainly influenced by the age of the animal [76]. The progressive transition from a simple unstable community into a complex and stable adult-like community has not only been described in production animals but also in rabbits [77], foals [78], squirrels [79], giant pandas [80] and rhesus macaques [81,82]. Increasing attention has also been focused on the microbial succession of the GIT of pets [83,84]. Especially the impact of macronutrient ratios of prenatal and pre- and post-weaning (commercial) diets (e.g. the protein/carbohydrate balance) has been studied. For example, lactic acid bacteria, members of the *Atopobium* group and bifidobacteria were dominant in the kittens' gut, but the latter bacterial group decreased significantly when the kittens were fed a high-protein diet post weaning [83,85]. Consequently, the type of petfood given to our companion animals may already induce changes early on in the microbial composition of growing kittens and pups.

2.2.2. In diversity, stability and resilience, there is strength

The phylogenetic architecture of an adult gut microbiome has co-evolved with their different mammalian hosts [86], holds an immensely complex diversity, varies considerably between individuals and displays a long-term stability at its core [87–89]. This stability can be defined as resilient stable states of the microbial community. In this definition, stability refers to the ability of the community to resist disturbances, stochasticity and temporal dynamics of individual microbes, whereas resilience refers to the capacity of the community to return to this equilibrium following perturbations [88,90]. These key features determine a healthy microbiota in a state of normobiosis and homeostasis, *i.e.* an ecological landscape in which micro-organisms with potential health benefits are in balance with potentially harmful ones [91].

As consistently shown by the finding of interindividual differences [88], an ideal community composition does not seem to exist. Yet, a core set of key players can be found in most mammalian GI ecosystems. Firmicutes (65.7%), Bacteroidetes (16.3%), Proteobacteria (8.8%) and Actinobacteria (4.7%) constitute the majority of the dominant bacterial phyla, followed by smaller fractions of Verrucomicrobia (2.2%) and Fusobacteria (0.67%). This percentual distribution results from an ensemble of 19 548 sequences acquired from faecal samples of humans and 59 other mammalian species, though numbers should be considered as indicative ranges [86]. The same study also revealed the powerful

prediction of faecal microbiota composition through diet (*i.e.* clustering herbivores, omnivores and carnivores apart) and gut physiology (*i.e.* separating fore- and hindgut fermenters and those with a simple gut). At family level, key players mainly belong to *Ruminococcaceae*, *Lachnospiraceae*, *Clostridiaceae*, *Eubacteriaceae*, *Erysipelotrichaceae*, *Bacteroidaceae*, *Lactobacillaceae*, *Coriobacteriaceae*, *Bifidobacteriaceae* [92,93]. It was recently proposed that humans may be divided, independent of gender and nationality, into three enterotypes distinguished primarily by levels of *Bacteroides*, *Ruminococcus* and *Prevotella* [94], although it appears that long-term food preferences may contribute to enterotype formation [95]. At a finer phylogenetic level, identification of genera and species leads to a plethora of individual variations on the main themes. Decreased taxonomic diversity and/or compositional changes, especially at finer scale phylogenetic level, have already been linked with numerous diseases, e.g. type 1 diabetes, obesity, irritable bowel syndrome, inflammatory bowel diseases, atherosclerosis, cystic fibrosis, autism, allergy and asthma [96–98]. These qualitative and quantitative detrimental changes in the intestinal microbial communities and their corresponding metabolic activity have been defined as dysbiosis, but whether the observed dysbiosis is either cause or consequence of the disease remains largely unclear [96]. Elucidation of these species and their mutualistic interactions can however yield proper insights for personalized therapies and pin each individual on the microbial map [99–101].

Characterizing diversity in phylogenetic terms has revealed a core gut microbiome with large interindividual variability, but in terms of genetic potential, individuals seem to harbour similar genes encoding specific metabolic functions or clusters of orthologous groups, thus providing evidence for a functional core microbiome [102]. Consequently, the GIT supports a high level of functional redundancy, meaning that different microbial constellations are able to sustain comparable functions. Although a healthy adult gut microbiome is still far from being defined in terms of compositional and functional diversity, insufficient diversity or evenness (*i.e.* distribution of relative abundances of species) in the microbial community structure appears to diminish its ability to withstand perturbation. Again, this emphasizes the significant role played by host-associated microbiota in mammalian health.

2.3. Microbial functionality in the mammalian gut

As Louis Pasteur once said "*Life would not long remain possible in the absence of microbes*". In fact, we could get by just fine, for a few days, and although life as an entity would endure, its quality would become tremendously bad. For a long time, the importance of the intestinal microbiota was neglected in favour of the enteropathogens causing disease. In the last few years, we've taken amazing leaps forward in our understanding of the microscopic world within us and have discovered a myriad of beneficial microbiota-associated functions in host physiology. The great mansion of our gut provides an attractive environment for microbes through a rich and continuous supply of nutrients and favourable anaerobic conditions. In turn, the gut microbiota assists in maturation of the GI epithelium and mucosa, priming of the immune system and a wide range of metabolic activities within and outside the GIT.

2.3.1. Shaping the host's immune system

The mammalian immune system is composed of a complex network of innate and adaptive components. The innate immune system acts immediately through a network of pathogen recognition receptors of microorganism-associated molecular patterns (MAMPs; e.g. lipopolysaccharides, teichoic acids, flagellins, peptidoglycans and lipoproteins). Subsequently, signalling cascades in the host are activated to eliminate the recognized microorganisms by production of chemokines and antimicrobial factors (e.g. antimicrobial peptides and secretory IgA). The adaptive immune system reacts in delay and relies on clonally selected lymphocytes with diverse receptors, capable of recognizing a huge variety of antigens and eliciting specific and memory responses [103]. The early colonizers in the GIT set tone for distinguishing microbial friends or foes. Relative immaturity of the neonate immune system at birth creates a tolerogenic environment in which the first recognitions of MAMPs are mediated by receptors that are impaired in the production of inflammatory mediators and promote microbial colonization in a unique way. Early responses to microbial ligands such as lipopolysaccharides condition gut epithelial cells to become hypo-responsive to subsequent receptor stimulation [104–106]. Studies in germfree mice have also revealed the critical role of the gut microbiota in intestinal epithelial cell and lymphoid structure development as they exposed smaller Peyer's patch size, reduced number of $CD4^+$T cells and IgA-producing plasma cells, compromised epithelial cell maturation and angiogenesis [107–109]. Furthermore, the resident microbial communities are an integral and essential part of the array of mucosal defence barrier mechanisms operating in the GIT through direct and indirect colonization resistance against allochthonous microorganisms. Direct colonization resistance refers to the competition with enteric pathogens and transient bacteria for attachment sites and nutrients and the production of compounds inhibiting their growth. Indirect or immune-mediated colonization resistance refers to the intestinal microbiota activating innate and adaptive immune responses in the host that in turn target pathogenic bacteria by the production of antimicrobial and pro-inflammatory factors [110].

2.3.2. Tailoring the host's behaviour

Our chorus of microbes gets a say in who we become and how we feel through a complex gut-brain crosstalk defined as the microbiota-gut-brain axis (MGB). This bidirectional communication system involves multiple endocrine, immune and neural pathways including the vagus nerve, the hypothalamic-pituitary-adrenal axis, cytokines produced by the immune system, tryptophan metabolism and production of short-chain fatty acids (SCFA) [111]. Some bacteria also have the capacity to produce neurotransmitters and neuromodulators such as gamma-aminobutyric acid (by *Bifidobacterium* and *Lactobacillus* spp.), norepinephrine (by *Escherichia*, *Bacillus* and *Saccharromyces* spp.), dopamine (by *Bacillus* spp.) and acetylcholine (by *Lactobacillus* spp.). These neurotransmitters may stimulate gut epithelial cells to release molecules that modulate neural signalling [112]. Many experimental approaches to elucidating the MGB axis have so far only included rodents, but these experiments have already shown psychological effects upon changes in the gut microbial composition. For example, an

increased anxiety-like behaviour was associated with the absence of intestinal microbiota in mice [113] and swapping the microbes between two genetically distinct strains of mice displaying different anxiety-like behaviours also swapped their anxiety level [114]. The intestinal microbiota may also be able to shape our minds as we develop and autism spectrum disorders have been linked to gut microbial disturbance [115]. Another interesting hypothesis is the impact of microbes on the development of sociability and group living. Lombaro (2008) suggested that the need to obtain certain microbes from conspecifics is a driving force in the evolution of social behaviour [116], a concept raised earlier by Troyer (1984) who stated that the need for cellulolytic microbes influenced the social behaviour in herbivores [117]. Also in non-human primates, specific social behaviour possibly related to microbial exchange (e.g. mother-infant bonding, mouth-to-mouth interaction) has been studied [118]. Particularly in mammals, lactation is considered to have a profound influence on behavioural and gastrointestinal development. Early maternal separation produces long-term alterations in behaviour, the hypothalamic-pituitary-adrenal axis and the gut microbiota. The stress-response raises cortisol levels that can alter gut permeability and barrier function, and thus contribute to changes in gut microbiota composition [119]. Experimental treatment of mice with probiotic *Bifidobacterium infantis* can normalize stress-related behaviour [120]. A human intervention study showed reduced cortisol levels after treatment with a probiotic cocktail of *Lactobacillus helveticus* and *Bifidobacterium longum* [121]. Taken together, cumulating evidence signals interesting possibilities for future research on the role of microbial genes in neuronal function and behavioural disturbances.

2.3.3. Modulating the host's xenobiotic metabolism

Owing to its sheer number of genes (2 up to 20 million microbial genes vs. 20 000 human genes) [102], it is hardly surprising that the gut microbiome can metabolize many compounds in ways that mammals cannot. As such they are involved in xenobiotic metabolism, which is the biochemical modification of xenobiotics *i.e.* foreign compounds including therapeutic drugs and diet-derived bioactive compounds. In mammals, the liver is the major site for oxidative and conjugative conversions, but relevant biotransformations occur in the gut as well [122]. This is of particular relevance to clinical practice since gut bacterial metabolism can affect pharmacokinetics of administered drugs.

Gut microbial modulation of xenobiotic metabolism can occur via direct mechanisms including the activation, detoxification or inactivation via direct binding to bacterial cells of xenobiotics. The indirect mechanisms involve microbial manipulation of host physiology through participation in the enterohepatic cycling, competition with xenobiotics for enzyme binding sites, production of metabolic pathway intermediates and/or stimulation of immune responses [123]. Although the majority of drugs are rapidly and completely absorbed in the upper GIT and have minimal contact with intestinal bacteria, over 40 therapeutic and diet-derived bioactive compounds are known to undergo direct or indirect microbial modifications. The importance of such modifications became clear after a case in Japan in 1993 when the promising antiviral drug sorivudine was introduced. Afterwards, it was discovered that sorivudine can be transformed by gut microbiota into (E)-5(2-bromovinyl)uracil which in turn can inhibit

the metabolism of an anti-cancer drug 5-fluorouracil. Co-administration of sorivudine with oral 5-fluorouracil prodrugs was reportedly responsible for the death of 18 patients [124]. In this respect, not only gut microbiota-mediated xenobiotic metabolism warrants further research, but also this novel type of gut microbiota-mediated drug-drug interactions. Antibiotics, in particular, can dramatically affect other drugs' metabolism by the gut microbiota in either a synergistic or antagonistic way. For example, Levamisole is an anthelmintic drug in veterinary medicine that is metabolized in the GIT to thiazole ring-opened metabolites by bacteria such as *Bacteroides* and *Clostridium* spp. Combined therapy with tetracycline has shown stronger biological effects because the antibiotic inhibits its metabolization by gut bacteria. The efficacy of the synthetic non-digestible disaccharide lactulose which is used for treatment of hepatic encephalopathy and chronic mega-colon in cats and dogs, on the other hand, is dependent on gut bacterial metabolism. *Lactobacillus* and *Bacteroides* spp. metabolize lactulose to fructose and galactose, which are then further fermented to lactic and acetic acids that lower the GI pH, draw fluid into the colon and inhibit the absorption of ammonia into the blood. The net effect is the reduction of blood ammonia concentrations and softening of the stool, all of which may be impaired by co-administration with antibiotics [125]. Given the interindividual differences in the gut microbiome, variations in xenobiotic responses are seemingly endless. Many challenges and opportunities are ahead of us to evolve to a microbe-focused medicine and to predict how a given individual would respond to a xenobiotic agent.

2.3.4. Supporting the host's digestion

The mammalian gut microbiota is known to be commensal, which in Latin means 'sharing a dining table', not a bad designation at all given the fact that our inner critters are feeding on the food leftovers after digestion by the host. Undigested and indigestible food components reach the gut microbiota in the colon after a relatively quick passage through the main sites of enzymatic digestion *i.e.* the oral cavity, stomach and small intestine. The transit time is highly dependent upon type of dietary compound and mammalian specific gut anatomy and physiology [126,127]. Colonic microbial fermentation of these dietary-derived components results in bacterial metabolites, the ensemble of which is referred to as the metabolome. The actual number of metabolites produced by diverse gut bacteria is not known and the number of metabolites that have the ability to exert an effect on the host is also unknown, but effects for most of them are assumed to be limited considering their transient passage at very low concentrations [128]. However, interindividual variations in metabolic signatures between healthy and/or diseased mammals suggest their sheer impact on host health [129]. These variations result from variable metabolite concentrations rather than from variable metabolite composition alone, thus supporting the hypothesis of a shared functional core microbiome across mammals [102]. The type of dietary substrates that are available to the gut microbiota largely determines the metabolic output (*i.e.* which metabolic pathways are used for fermentation) and in turn influences the gut microbial composition (*cf.* enterotypes reflecting habitual dietary intake) [95,130].

One of the preferred energy sources for the gut microbiota in mammals is dietary fibre which can neither be defined as a single chemical entity nor group of related compounds. Numerous definitions have been proposed, but all identify dietary fibre as carbohydrate polymers and oligomers materials that escape digestion in the small intestine and pass into the large intestine, where they are slightly or nearly completely fermented. Most scientists agree on the inclusion of indigestible non-starch polysaccharides, oligosaccharides, lignin and resistant starches [131]. However, definitions considered dietary fibre from plant origin only. Recently, the definition in the CODEX Alimentarius [132] has been broadened to "all sources of edible carbohydrate polymers that are found naturally in the consumed food, that can be obtained from food raw material or are synthetic in origin and are indigestible by human small intestinal enzymes', which may thus include compounds from animal origin. Dietary fibre *per se* and their fermentation products contribute to many physiological benefits associated with their consumption.

Main fermentation end-products of saccharolytic metabolism are short-chain fatty acids (SCFAs), primarily acetate, propionate and butyrate and gases (H_2, CO_2, H_2S and CH_4). Produced at lower concentrations are other organic acids such as valerate, lactate, succinate, malonate, caproate, fumarate and formate [128,133]. The main SCFAs (*i.e.* acetate, propionate and butyrate) provide approximately 2% of the energy requirements in carnivores, 10% in humans, 20-30% in omnivorous or herbivorous animals and up to 80% in ruminants. Moreover, their absorption might be vital under conditions of food scarcity [133,134]. SCFA absorption in the gut appears to be mostly passive and increases linearly with corresponding decreases in pH or increases in concentration [134]. The total amount of SCFA in the proximal human colon is estimated to range from 70 to 140 mM and falls to 20 to 70 mM in the distal colon, with less than 5% of bacterially derived SCFA appearing in the faeces due to colonic uptake [135]. SCFA production in mammals is in the order of acetate > propionate > butyrate, and appears in faeces of humans in approximately 60:20:20 molar ratio. The pH-lowering effect of the SCFA production is one of the many health-promoting effects because an acidic pH prevents overgrowth of pathogens, increases absorption of minerals (e.g. Ca binding to free bile acids and fatty acids), and reduces absorption of ammonia by protonic dissociation ($NH_3 + H^+ <-> NH_4^+$) [136]. Butyrate is a preferred fuel of the colonic epithelial cells and plays a major role in regulation of cell proliferation and differentation, whereas propionate and acetate reach the liver by the portal vein and exert more systemic effects (**FIG 2.1**). In addition, metabolic cross-feeding between bacteria is likely to play a role in substrate breakdown and butyrate production. Cross-feeding on breakdown products from primary polysaccharide degraders can benefit oligosaccharide-utilizing bacteria and may result in the production of SCFAs. For a number of bacterial species (e.g. members of clostridial cluster XIVa), another mechanism of cross-feeding is observed in which acetate and lactate produced by other bacteria function as key intermediates for butyrate production [137,138].

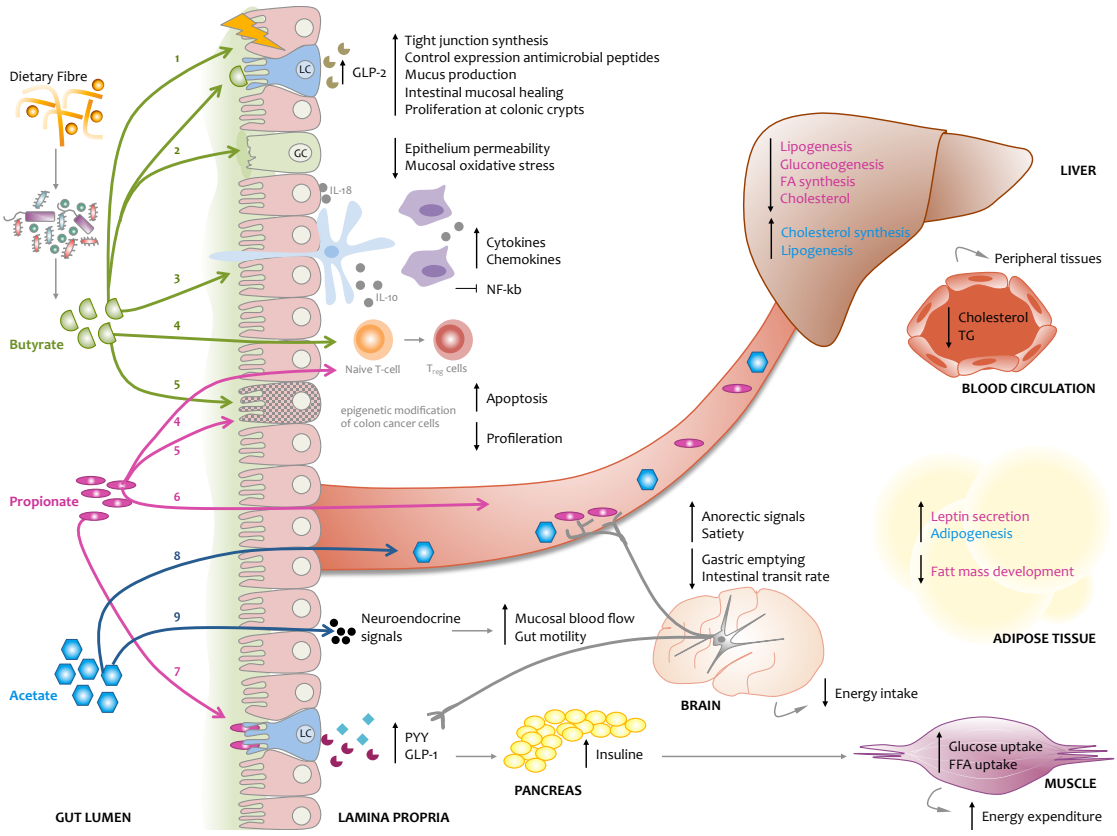

FIG 2.1 | Potential local and systemic health effects attributed to acetate, propionate and butyrate. These short-chain fatty acids result in particular from microbial fermentation of dietary fibre in the colon. Butyrate is (1) the primary energy source for enterocytes and effectuates mainly local effects in the gut: (2) intestinal barrier enforcement, (3) anti-inflammatory effects, (4) immunomodulation and (5) tumor suppression. Propionate reaches the liver by the portal vein and effectuates systemic effects such as (6) lowering serum cholesterol and triglycerides (TG), (7) regulate appetite and improve body condition score. Acetate effectuates mainly systemic effects by (8) induction of cholesterol synthesis and (9) stimulation of gut motility. Potential tumor suppression effects in peripheral tissues (e.g. breast) have also been attributed to both propionate and acetate. It must be stressed that not all effects are yet validated *in vivo*. (FA) fatty acids, (FFA) free fatty acids, (GLP-1 & GLP-2) glucagon-like peptide-1 and peptide-2, gut hormones secreted by L-cells, (PYY) peptide tyrosine tyrosine hormone secreted by L cells; (LC) L-cell; (GC) goblet cell producing mucin. [128,136,139–141]

The amount of protein and amino acids entering the colon depends on the protein content of the ingested food, the protein digestibility and the amount of enzymatically indigestible carbohydrates. Fermentation of proteins by proteolytic bacteria yields a much wider range of metabolites compared to dietary fibre fermentation including branched-chain fatty acids (BCFAs; e.g. isovalerate and isobutyrate), ammonia, hydrogen sulphides, polyamines, indolic and phenolic compounds as well as nitric oxide, nitrite and N-nitroso compounds [128]. Overall, these metabolites are known for having detrimental effects, potentially involved in the aetiology of colorectal cancer and ulcerative colitis in humans [142]. For example, *in vitro* studies have confirmed the (geno)toxicity of individual protein fermentation metabolites such as ammonia and hydrogen for epithelial cell metabolism and barrier function (e.g. sulphide prevents oxidation of butyrate in colonocytes and induces DNA damage). Previous studies have suggested a potentially harmful effect of a high protein intake (e.g. red meat may induce more genetic damage as compared with white meat or whey protein), which was supported by epidemiological human studies associating high red meat intake and the risk of developing adenomas.

However, these associations are weak in magnitude with no clear dose-response patterns [143]. Other studies even found no significant associations suggesting possible interplay with other risk factors (e.g. lifestyle). A more recent report suggested that it is not the high protein intake but the high concentration of haem in red meat that is associated with the risk of developing colorectal cancer [144]. In October 2015, the International Agency for Research on Cancer concluded that there is sufficient evidence to classify consumption of processed meat as 'carcinogenic to humans' and consumption of red meat as 'probably carcinogenic to humans' [145]. The effects of metabolites resulting from protein fermentation are presumably rather complex and depend on various factors such as their luminal concentrations or absorption, the duration of the colonic stasis, the detoxification potential as well as the adaptive capacities of the epithelial cells to utilize these metabolites. For example, polyamines (e.g. putrescine, cadaverine, tyramine, histamine, spermidine) have been implicated to have deleterious effects on postprandial motility and their involvement in malignant cellular transformation, but are also known to be essential for gut maturation and fluid secretion by colonic crypts [146]. Moreover, although SCFA are the major end products from carbohydrate fermentation, they are also produced from amino acids by reductive deamination. Acetate can be produced from glycine, alanine, threonine, glutamate, lysine and aspartate, whereas butyrate can be produced from glutamate and lysine, and propionate can be synthetized from alanine and threonine [146].

Host-derived substrates such as pancreatic secretory products, desquamated enterocytes, glycans and mucus proteins are also metabolized by gut bacteria. For example, the specialized mucin degrader *Akkermansia muciniphila* seems to be crucial for initial breakdown of mucins and modulation of mucosal immune responses [147]. Furthermore, gut bacteria are vitamin suppliers to the hosts because most of them lack the biosynthetic capacity for many vitamins. Vitamin B_{12} is known to be exclusively produced by microorganisms (e.g. lactic acid bacteria) but precursors for many others are also supplied [148].

Taken together, the intestinal microbes are in many ways the gatekeepers of mammalian metabolism. They have the potential to influence what we can eat, how many calories we derive from it, what nutrients and toxins we are exposed to, how we feel and how clinical therapies affect us.

2.4. Tools to decipher gut microbial diversity

"The key to taking the measure of biodiversity lies in a downward adjustment of scale. The smaller the organism, the broader the frontier and the deeper the unmapped terrain." (Wilson EO) [149]

2.4.1. Measures of species diversity

"Diversity per se does not exist" claimed Hurlbert (1971) and he suggested the term to be abandoned because of the multiplicity of meanings and interpretations attached to it [150]. Eberhardt (1969) believed that diversity stands for a confusion of concepts, definitions, models and measures [151]. One

may define diversity as different kinds distinguished by their differing characteristics of morphology, physiology and metabolism, by their various ecological distributions and activities, and by their distinct genomic structure, expression and evolution [152]. However, compared to large multicellular organisms, the recognition of differences and similarities among unicellular microbial organisms is compounded by the fact that structures of microbial communities are highly skewed such that a few kinds are abundant, and many are rare [153]. Consequently, preferential targeting of abundant populations and detection thresholds may significantly confound the outcome of diversity analyses (see also 2.4.2 and 2.4.3). Ecologists have partitioned diversity on three scales. Alpha diversity is the within-community diversity, while among-community diversity is referred to as beta diversity and the diversity across a landscape as gamma diversity [154]. Commonly used parameters to express diversity include the number of species (*i.e.* species richness) and the relative abundance of various species (*i.e.* species evenness) [155]. As the extent of microbial diversity is beyond practical calculation, diversity is estimated using various mathematical approaches (e.g. diversity indices and rarefaction curves) which take into account species richness and/or evenness [156–158]. These species-based measures have more recently been complemented by divergence-based measures which take into account the divergence between pairs of organisms, including sequence distance, phylogenetic distance, topological distance, and taxonomic distance (**TABLE S1**) [157]. Widely used diversity indices are the Simpson's index and the Shannon's index. The former reflects the probability that any two organisms randomly drawn from an infinitely large community belong to the same species, whereas the latter quantifies the uncertainty of predicting the next species to be found in the community [153].

Although one could debate the merits of diversity indices [159], the reality is that they are commonly used and imply a consensus on the discrete nature of 'kinds', meaningful units of diversity which should be properly identified in a sample or dataset. Historically, diversity measures relied on the species as fundamental unit for calculations. However, trying to find a unifying concept for classification of organisms has led to at least 20 proposed species concepts. The most familiar of these is the Biological Species Concept introduced by Ernst Mayr, who proposed that species are groups of actually or potentially interbreeding natural populations that are reproductively isolated from other such groups [160]. Although this concept has evolved, it remains problematic for the classification of bacteria, characterized by asexual reproduction and horizontal gene transfer across species. Consequently, the species concept for bacteria has sparked debate among microbiologists, leading to again numerous concepts [161]. Species delineations in bacteria are based on phenotypic, chemotaxonomic and genotypic traits. Bacterial isolates that have ≥70% genome DNA-DNA hybridization relatedness under standardized conditions with a melting temperature ≤5°C are assigned to the same species. Additional requirements are that a certain degree of phenotypic consistency is observed between the isolates and with the advent of sequencing of small subunit RNA genes, that they share ≥98.65% 16S rRNA gene sequence identity [162,163]. However, the congruence between values from DNA-DNA hybridizations and 16S rRNA gene sequences is highly variable in different lineages, as evidenced by the description of

different species exhibiting very high (>99%) 16S rRNA gene sequence similarity [161]. New and superior tools for delineating bacterial species are provided by the rapidly increasing availability of whole genome sequences. Average nucleotide identity (ANI) is such a genome sequence-derived parameter that represents a mean of the similarity values between homologous genomic regions shared by two bacterial genomes. It is proposed that ANI values of 95-96% equate to a DNA-DNA hybridization value of 70% as a boundary for species delineation [163,164]. The bacterial species concept has been intensively reviewed in recent years [165–168], but in the end one should also consider the fact that adequacy of characterization of a bacterium is a reflection of a snapshot in time, and that it constantly evolves in parallel with the design of next-generation taxonomic techniques. An appropriate taxonomic framework to study diversity and evolutionary relationships among organisms (*i.e.* phylogeny) based on marker genes such as the 16S rRNA gene (see Box II) remains crucial, but the advent of high-throughput sequencing techniques generates large sequence databases based on marker gene surveys which require also another framework to describe diversity which is not necessarily cultured or culturable. To this end, operational taxonomic units (OTUs) or phylotypes have been defined as a theoretical framework for relating sequence differences to discrete taxonomic entities. These are surrogates for a taxonomic rank (e.g. species) and are typically based on a specified nucleotide identity consistent with the conventional bacterial species threshold (e.g. ≥ 97% identity for 16S rRNA marker genes). By defining OTUs, sequence data are organized and reduced, and several diversity indices can be applied to describe community diversity.

BOX II – the 16S rRNA gene, desired and despised

Carl Woese and colleagues pioneered the use of ribosomal RNA genes (rRNA) for phylogenetic studies in the early 1970s and revolutionized bacterial phylogeny and taxonomy. The three types of rRNA genes in prokaryotic ribosomes are classified as 5S, 16S and 23S, have sequence lengths of about 120, 1550 and 3300 nucleotides, respectively, and are typically organized into a gene cluster expressed as single operon in members of the domain Bacteria. The 16S rRNA gene has been chosen over its companions because it is more easily and rapidly sequenced compared to the 23S rRNA gene and contains more phylogenetically informative sites compared to the 5S rRNA gene. Moreover, it is characterized by its universal presence in prokaryotes, its high functional constancy (i.e. lateral gene transfer seems minimal) and its specific properties as a phylogenetic marker (i.e. enough variability to infer phylogenetic differences and enough conserved regions to allow their amplification) [183,482,483]*. The 16S rRNA gene is a structural part of the 30S ribosomal small subunit and is composed of eight highly conserved regions interspersed with nine hypervariable regions, which serve as primer docking sites and as phylogenetic markers, respectively. These hypervariable regions have a high degree of interspecies variability. Its wide adoption for phylogenetic analysis has resulted in extensive 16S rRNA gene sequence databases which also allow comparative analyses, and ultimately, identification of unknowns. However, the presence of multiple rRNA operons (up to 15 copies) in the bacterial genome of some species may interfere with estimates of species richness (i.e. multi-operon effect). Furthermore, the gene does not reflect the remaining functional genome content* [484] *and its resolving power is insufficient to guarantee correct delineation of bacterial species within specific groups of closely related species (e.g. lactic acid bacteria). These limitations have led to alternative targets (i.e. housekeeping genes encoding for bacterial housekeeping functions) with higher sequence polymorphism distributions such as the cpn60, recA and rpoB genes* [416,485,486]*. However, none of these genes are without limitations and the lack of comprehensive databases hampers their general implementation. Another alternative is the 16S-23S rRNA intergenic spacer region, which is variable in length, sequence and copy number even within closely related taxonomic groups, but may underestimate diversity due to similar region length between unrelated species or overestimate diversity due to multiple copy numbers* [487]*. With the advent of high-throughput sequencing techniques, a shorter region of the 16S sequence must be selected to act as proxy. The lack of consensus on a single 'best' region results in different variable regions sequenced and hinders comparisons among studies* [172]*. Nevertheless, at present, 16S rRNA gene sequence analysis still provides the most stable and satisfactory framework to conduct phylogenetic analyses.*

2.4.2. Getting it right from the start

A chain is only as strong as its weakest link. As such, each study design depends on well-considered choices along the way to achieve the goal set forth. Depending upon the techniques selected, different choices are to be made. For instance, prior to the application of many molecular methods lies the choice of sample, sampling, storage and processing, as well as the choice of DNA extraction method, and eventually primers and PCR amplification conditions.

Owing to practical and ethical concerns, especially in animals, most of the knowledge of the mammalian GI microbiome is obtained from faecal samples, which are considered to provide a good readout of the luminal microbiota in the distal GI tract (*i.e.* distal colon and rectum) [169]. However, it is clear that more invasive sampling is warranted to study mucosa-associated communities. The optimal approach for sampling these communities has not yet been defined, but includes mucosal biopsies and/or less invasive mucosal brushing [170]. Although it has been suggested that differences between groups are more apparent upon pooling of faecal samples, it is generally not recommended, given the variability in 16S rRNA lineages observed among samples [169,171,172]. Faecal swabs are routinely used in clinical settings to detect enteropathogens (mostly through culture-based methods), but their use for characterizing community diversity might be arguable, especially when faecal material is rather heterogeneous and includes undigested dietary particles (e.g. plant or animal components) [173]. Upon defaecation, the viability and structure of the faecal microbial communities are menaced by the increase in oxygen levels and the loss of nutrients from the gut environment, which implies immediate preservation under the right conditions and assurance of anaerobic conditions if collection is aimed at culturing. Even a delay of several hours during which samples are kept at room temperature or 4°C may affect faecal microbial composition and bacterial DNA integrity [174]. In contrast, other studies showed no significant influence on phylogenetic structure and diversity of faecal communities after 24h storage at 4°C [173] or even 14 days at room temperature [175]. Common practice is to store faecal samples immediately after defaecation at -20°C and preferably at -70/-80°C for long-term storage. Direct freezing increases the potential for additional analyses (e.g. metatranscriptomics and metaproteomics), whereas thawing during transport should be avoided, as well as repeated thawing and freezing of samples during storage [173]. If direct freezing is not feasible, immediate cooled storage at 4°C for a maximum of 24h is preferred. However, it has been reported that freezing of faecal samples prior to DNA extraction may affect the Firmicutes to Bacteroidetes ratio, a frequently used biomarker determined by downstream quantitative PCR analyses [176]. To minimize potential bias resulting from faecal sampling, storage and handling, a standard operating procedure should be applied for each sample within the study design.

Next, obtaining maximum yield of high quality DNA by an efficient and reproducible extraction method is of paramount importance to reflect the 'true' microbial diversity of a sample. The most crucial step in the DNA extraction process, cell lysis, can be tackled with various enzymatic, chemical and/or

mechanical (e.g. beads) procedures, using commercial kits, in-house laboratory protocols, or a combination thereof, each with their own potential biases [177–180]. The different sensitivity of GI microbial key players to these procedures is particularly challenging. Different cell wall structures of Gram-negatives (e.g. members of the phylum Bacteroidetes) and Gram-positives (e.g. members of the phylum Firmicutes) make it almost impossible to extract DNA from all constituting species with the same efficiency. Rigorous mechanical lysis assures higher bacterial numbers and diversity of clostridial and actinobacterial populations, whereas harsh DNA-extraction conditions (e.g. repeated bead beating and extended heat lysis) may lower numbers of Bacteroidetes species [181]. The principle of the DNA extraction procedure should therefore always be kept in mind when interpreting microbial community diversity measurements. Other important considerations include the variable microbial loads of different GI samples, the heterogeneous microstructure (e.g. undigested material with biofilms) and the presence of PCR inhibitors (e.g. bile salts, plant polysaccharides, phenols, degradation products of haemoglobin, immunoglobin G). DNA-extraction procedures should remove these inhibitors and at the same time avoid residual high concentrations of potential PCR inhibitors introduced by the method itself (e.g. sodium deoxycholate, sarkosyl, EDTA, phenol, sodium dodecyl sulphate) [182,183].

Many molecular methods for community analysis rely on the amplification of DNA of all microorganisms present in a mixed microbial sample, and a number of primers have been designed that anneal to conserved regions of targeted marker genes. Although commonly referred to as 'universal' primers, they are unlikely to target 'all' prokaryotes and primers should be evaluated for universality and/or specificity. In addition, amplification is prone to biases which can be categorized as annealing biases (*i.e.* associated with primer annealing) or amplification biases (*i.e.* those which occur during PCR cycling) [183,184]. As a result, the view on community diversity may be distorted towards differential or preferential amplification of target genes leading to under- or overrepresentation of single species or even whole groups.

Lozupone and co-workers recently addressed the impact of technical variability by comparing 16S rRNA gene sequences generated from diverse studies of the human microbiota [92]. Their results showed that for comparisons within faecal samples, compositional differences associated with age and geography/culture were greater than those driven by the experimental protocols used. However, when comparing only faecal samples from adult Western populations, clustering occurred primarily by study. This indicates that differences in experimental protocol, including choice of PCR primers/16S rRNA region targeted, DNA extraction protocol and sequencing platform can be associated with differences in the observed diversity. In a joint effort to optimize data quality and comparability in the human microbiome field, the International Human Microbiome Standards (IHMS) project was launched to serve as a benchmark for standardized sample collection, processing, DNA sequencing and data analysis (www.microbiome-standards.org). This cohort is partnering with the Microbiome Quality Control project (MBQC) in which methods for measuring the human microbiome are evaluated to improve the state-of-the art in the workflow from sampling to microbiome data processing (www.mbqc.org).

2.4.3. From culturing to culture-independent community profiling

A non-exclusive overview of methods used in GI microbial diversity analyses complemented by those that focus on the functional role of the GI microbiome is presented in **FIG 2.2**. Methods implemented in this thesis are highlighted. In the following sections, the inherent advantages and disadvantages of a selection of these techniques are briefly discussed.

Until some years ago, the vast majority of GI microbial diversity was inaccessible or largely underestimated by ***culture-dependent methods***. Prior to the introduction of molecular methods, the composition of a complex microbial community was determined by microscopy to determine major morphological differences followed by culturing to isolate bacterial species for phenotypic characterization [185]. Notwithstanding the advent of anaerobic culture techniques and the promising results obtained with co-culture experiments [186], culturing remains laborious and time-consuming. Furthermore, it is an approach hampered by the lack of knowledge on fastidious growth conditions of many GI bacteria and the challenging recovery of microorganisms who can enter into a viable but non-cultivable state when exposed to various stresses [187]. Consequently, the view on the microbial diversity resulting from cultivation alone is biased and mostly incomplete. The advent of sequencing-based techniques spurred the development of culture-independent molecular tools at rapid pace and largely supplanted cultivation. On the other hand, the collection of pure isolates remains essential for characterisation of novel species, mechanistic studies of cell-cell and host-cell interactions and evaluating their potential use as probiotics and biotherapeutics. Moreover, recent research showed that the low coverage of the human gut microbiota in culture largely reflects a discrepancy of number of cultured isolates relative to the obtained sequence information, rather than any intrinsic unculturability. Thus, it is expected that many novel microorganisms can be discovered as a result from extensive culturing [188]. The novel concept of culturomics has renewed the interest in culturing. The strategy involves the use of multiple combinations of culture conditions mimicking the natural conditions within the gut (e.g. atmosphere, incubation temperature and time, medium composition) combined with rapid colony identification by mass spectrometry (MALDI-TOF MS) and 16S rRNA gene amplification and sequencing [189,190]. Culturomics can mitigate some of the limitations of molecular techniques by assigning functionality to specific microbial lineages and by expanding reference databases through sequencing whole genomes of cultured representatives of new species or functionally interesting isolates.

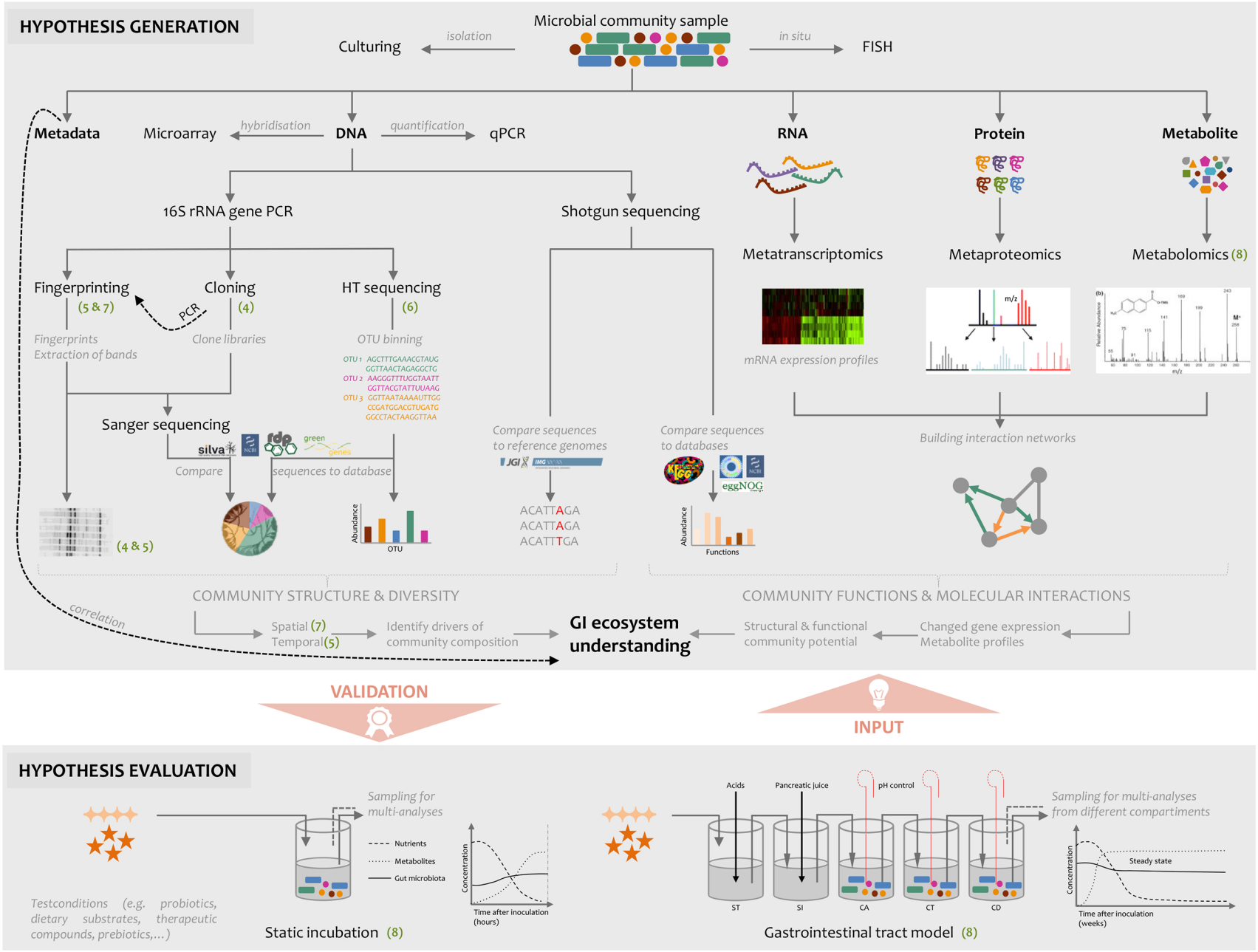

FIG 2.2 | Pathways to gut microbiome profiling. Integrating meta-omics to complement traditional methods and add to hypothesis generation and subsequent hypothesis evaluation with *in vitro* models. Techniques implemented in this thesis are marked with the corresponding chapter number. (ST) stomach; (SI) small intestine; (CA) colon ascendens; (CT) colon transversum; (CD) colon descendens

Culture-independent molecular tools for community analysis can be roughly categorized into non-sequencing based methods, including fluorescent *in situ* hybridization (FISH), quantitative PCR (qPCR) and community fingerprinting methods, and sequencing-based methods, including clone libraries and high-throughput metagenomic sequencing (see section 2.4.4). However, alternative classifications can be made depending on whether methods are 16S rRNA gene-based or not, assess community assembly and/or functionality or whether generated data are qualitative and/or quantitative. The combined use of methods has been advocated to combine strengths of various methods and mitigate their weaknesses.

Fluorescence in-situ hybridization (FISH) is an image-based technology that relies on the hybridization of target sequences in cells with fluorescence-labelled oligonucleotide probes. Subsequent coupling with epifluorescence microscopy and flow cytometry allows visualization and enumeration. This method thus provides valuable information on spatial distributions of bacterial cells and their association with specific tissues in different types of samples (e.g. ingesta, faeces, mucosal tissues). Furthermore, it enables identification of specific populations and community structure and determination of metabolically active members. Moreover, it does not need living cells and circumvents PCR biases. However, large drawbacks are its low-throughput capacity, potential problems with low probe specificity and target accessibility, its inability to detect unknown species and its lower enumeration efficiency compared to quantitative PCR [191,192].

Quantitative PCR (qPCR) or ***real-time PCR*** is a commonly used high-throughput PCR method allowing rapid and accurate quantification of bacterial targets in a large dynamic range (approximately six orders of magnitude). Three major approaches include quantification of (i) total bacteria within a community, (ii) specific groups of bacteria or (iii) individual species or strains. This powerful extension of conventional PCR relies on continuous recording (*i.e.* in 'real-time') of fluorescent signals associated with amplicon accumulation during each cycle in the PCR. Commonly used fluorescence chemistries include Sybr Green dyes and TaqMan probes and offer great sensitivity. However, the target specificity is determined by the design of the primers and the PCR conditions. Usually, 16S rRNA gene serves as target although single-copy housekeeping genes have been evaluated as well. During the exponential phase of the amplification, the amount of target amplified is proportional to the starting template. Next, gene numbers are then quantified using the C_t method. The C_t value is the baseline threshold at which fluorescence can be distinguished from background noise and is crossed by each sample at a different quantification cycle (Cq), depending upon the sample's initial target concentration. Absolute quantification of an unknown target template is determined by comparison of the C_t value against a standard calibration curve generated from initial known template concentrations for which the corresponding C_t values are plotted against the log of the initial copy numbers. This robust, highly reproducible and sensitive method has been used to track phylogenetic changes in GI samples across temporal and spatial scales under varying environmental or experimental conditions. Examples include the study of gut microbiota variations associated with age, antibiotic use and disease pathology (e.g.

obesity, autism, irritable bowel syndrome etc.) [193–197]. The method's initial inability to also detect changes at the functional level was countered by the implementation of reverse transcription analyses, offering a tool for quantification of gene expression. Despite its powerful and useful capacities, qPCR is unable to detect novel species and shares biases inherent to any PCR-based approach [198].

Another semi-quantitative and fast tool for phylogenetic identification of complex microbial communities are ***DNA microarrays***. These allow parallel analysis of DNA of thousands of genes or of the same gene from thousands of organisms in a single experiment. Lower-density microarrays have also been developed for various diagnostic applications. Specific microarrays address the human gut microbiota e.g. the HITChip designed to target 1140 species using 4809 overlapping probes, the HUGChip composed of 4441 probes targeting 66 families and the Microbiota array targeting 775 phylotypes [199–202]. Consequently, an important limitation is the inability to detect hitherto uncharacterized phylotypes and the variable stringency of hybridization. In addition, the utility of human specific arrays in the study of GI communities of other mammals requires further determination [203].

A last group of non-sequencing based methods includes community profiling through sequence-dependent electrophoresis (SDE) of the 16S rRNA gene or the 16S-23S intergenic spacer (IS) region. The latter region is the target in ***automated ribosomal intergenic spacer analysis (ARISA)***, a method seldom used in GI microbial diversity studies due to the lack of optimized primer sets and the paucity of IS sequence datasets. Recently, a new intergenic profiling technology has been proposed for analyses of clinical samples [204]. The 16S rRNA-based techniques have been commonly applied in the field of mammalian GI microbial ecology, and proven to be valuable for comparing GI microbial communities between different individuals, between different sites of the GI tract and for evaluating the effect of specific dietary regimes or therapies [205–208]. They have also been extensively used for monitoring population dynamics [206,209–211]. The SDE principle relies on the sequence-based electrophoretic separation of equally sized PCR products in a polyacrylamide gel containing either a linearly increasing gradient of a DNA denaturant ***(Denaturing gradient gel electrophoresis [DGGE])*** or a temperature gradient ***([temporal] temperature gradient gel electrophoresis [TGGE]/[TTGE])***. Alternatively, ***terminal restriction fragment length polymorphism (T-RFLP)***, which differentiates microbial community members based on terminal restriction fragment (TRF) sizes, has been successfully applied to study diversity and dynamics of the GI microbial ecosystem [208,209]. Mixed DNA templates are amplified with fluorescently labelled primers and subsequently digested with selected restriction enzymes to generate TRFs. These are then resolved according to fragment size by automated capillary electrophoresis [183,212].

SDE fingerprinting tools rapidly provide a snapshot of an unknown community composition at a fraction of the cost associated with sequencing techniques. Furthermore, they allow medium- to high-throughput of samples in a reproducible manner. However, they do not allow in-depth analysis of the complex GI microbial communities. Apart from biases inherent to PCR, the multi-operon effect of the

target 16S rRNA gene increases risk of overestimating the 'true' diversity, whereas co-migration of amplicons and the lack of sufficient polymorphic regions to differentiate closely related species may lead to underestimation of the diversity. In addition, it should be kept in mind that fingerprints reflect mostly the predominating members (*i.e.* taxa representing more than 1% of the total community), though this depends on the efficiency of DNA extraction and the absolute or relative abundance of bacterial groups in the sample. Another drawback is the fact that the tools do not deliver direct taxonomic information. However, they can provide phylogenetic information when used in concert with sequencing-based techniques. As such, DGGE bands can be excised from the fingerprint profiles and sequenced, or band position analysis of unknown bands with bands from known reference sequences can be performed [212]. Recently, the role and usefulness of fingerprinting methods in contemporary microbial ecology was questioned. Moreover, their low sensitivity towards less abundant taxa and their inability to identify accurately bacterial taxa to the species level was criticized. It is evident that a fingerprinting technique cannot provide a complete inventory of all bacterial taxa present in a sample and it is questionable if any technique will ever do so. Indeed, no single method is completely devoid of a potential bias. However, it is a matter of research questions asked and available tools. Despite their lower resolution, SDE-based fingerprinting methods are capable of detecting the same major spatial, temporal and treatment shifts in GI communities as is the case for 454 sequencing data, and still provide an independent and legitimate tool for the rapid generation of community patterns [213]. As Bent et al. (2007) aptly illustrated: 'Fingerprinting techniques are like prêt-a-porter clothes: dressing prêt-a-porter is maybe not as glamorous as being dressed by a top stylist, but it does not leave you naked' [214].

Sequence analysis of ***16S rRNA gene clone libraries*** is one of the earliest and most widely used sequencing-based methods providing an unparalleled level of phylogenetic resolution due to the long read lengths generated by Sanger sequencing technology (± 1000 bp) [215]. This method involves the construction of clone libraries from phylogenetic marker genes by ligation of purified amplicons into plasmid vectors and subsequent transformation into competent *Escherichia coli* cells. Transformed cells are identified through spread-plating onto a selective medium, and are subsequently picked up for Sanger sequencing of the inserts. The method's relative simplicity and limited infrastructure requirements combined with the Sanger sequencing accuracy and ability to sequence the near complete 16S rRNA gene has allowed its application in numerous studies on mammalian GI microbial diversity and provided a wealth of phylogenetic information and species diversity [216]. As with all PCR-based tools for community analysis, clone libraries are subject to PCR biases. Other important caveats are cloning biases and underestimation of the diversity due to the limited number of clones that can be processed for logistical reasons. Although clone library construction is time-consuming, labour-intensive and relatively expensive, it remains a useful tool for microbial diversity analysis [183].

2.4.4. Metagenomics for taxonomic and predictive functional profiling of microbial communities

Advances in sequencing technologies and bioinformatics, and a reduced cost per base, enable researchers to investigate the collective set of genes contained within a complex microbial community and assess the genetic and functional potential of a given microbiome in a high-resolution and culture-independent manner. Two distinct metagenomic strategies are used in this context: amplicon sequencing of pooled 16S rRNA PCR amplicons or shotgun sequencing of all DNA fragments present in a given sample. The ***16S rRNA gene-based metagenomic methods*** require amplification of one of the variable regions of the gene prior to sequencing using a specific platform of interest (e.g. Ion Torrent [Life Technologies], 454 [Roche Diagnostics], Illumina [Illumina Inc.]). Subsequently, the millions of 16S rRNA reads generated by high-throughput sequencing are clustered into OTUs on the basis of their degree of sequence identity and compared to several databases for taxonomic classification. This is a bioinformatic challenge for which multiple tools and platforms (e.g. MG-RAST, METAREP, BLAST, Mothur etc.) have been developed to handle data analysis [217–219]. Compared to shotgun metagenomic sequencing, storage of phylogenetic metagenomic data is manageable and the approach is more cost-effective. Important drawbacks apart from those intrinsic to the gene and PCR include the short read length that limits the precise identification at species level, the abundance of chimeric sequences and the potential underestimation of minor populations. Advances in bioinformatic analysis and increasing read length of Illumina sequencing allow, at least partially, these difficulties to be overcome [199]. In addition, the development of software (e.g. MG-RAST, PICRUSt) that imputes functions based on 16S rRNA profiles by associating these with annotated reference genomes allows predictive functional profiling [220]. However, this output is biased by the hypervariable region(s) sequenced and thus used for taxonomic assignment. In that context, ***shotgun metagenomic sequencing*** offers more potential to identify metabolic functions [221]. In shotgun sequencing, extracted community DNA is sheared in fragments and sequenced. The resulting reads are blasted against various databases including functional databases (e.g. KEGG, MetaCyc) to trace genes and allow for functional pathways reconstruction [217]. The downside of this method is the need for extensive sequencing reads to ensure sufficient coverage, which increases the storage burden of large datasets generated and complicates bioinformatic analysis. In addition, a large proportion of the reads may be left unidentified [199,222]. However, whole-metagenome shotgun sequencing can reveal strain-level variation, which may be required in studies where phenomena occur at the strain level (e.g. acquisition of antibiotic resistance genes) [223]. An important fundamental limitation of metagenomics is the inability to directly measure functional activity of a community. To fully understand the determinants of function, integration with other omics approaches is warranted.

2.5. Tools to decipher gut microbial functionality

2.5.1. Integrating functional omics approaches

The genomic content tells us what the community is capable of, but does not provide information on what the community is doing at a particular time point or in a particular condition. Triggered by this limitation, the use of high-throughput sequencing approaches has expanded to biological molecules other than DNA.

Metatranscriptomics involves sequencing of the total RNA present in a microbial community. Metagenomic and metatranscriptomic sequencing can be carried out in tandem and reveal changes in functional activity in response to perturbations. For example, the supplementation of a fermented milk product as a probiotic minimally affected microbial composition but significantly changed microbial gene expression [224]. Metatranscript studies of the human gut microbiome also revealed a large degree of inter-individual variability [225]. Technical aspects such as the enrichment of mRNA and its short half-life time make this approach logistically more challenging [222].

Metaproteomics relies on mass spectrometry-based shotgun quantification of peptide mass and abundance. It is a valuable tool to monitor bacterial as well as host proteins that allows an integrated analysis of host and microbial functions [226]. For example, depletion of proteins involved in the maintenance of epithelial integrity was observed in patients with Crohn's disease [227]. Hence, identification of protein changes associated with disease pathologies may lead to the discovery of potential biomarkers. However, the extraction of proteins from inferring compounds and membranes is technologically challenging, and the subsequent bioinformatics analysis is complex. Although proteomics offers possibilities to reveal host-bacterial interactions and thus confirm microbial function, they do not necessarily reflect the actual production of metabolites resulting from functional activity.

Metabolomics covers the detection of metabolites and other small molecules in microbial communities. It relies on chromatographic techniques to separate compounds, which are subsequently identified and quantified using mass spectrometry (MS) or nuclear magnetic resonance. Given the fact that host-microbe interactions are often mediated at the level of shared metabolite pools or metabolite exchanges, this is a crucial tool for understanding specific functional activities, distinguishing between healthy and diseased individuals and providing biomarkers of disease [221]. This has been illustrated by Wang *et al.* (2011) who were able to link the microbial conversion of a dietary metabolite phosphatidylcholine into trimethylamine N-oxide to atherosclerosis [228]. The targeted approach to metabolomics measures specific metabolites or class of metabolites, whereas the untargeted approach provides a broad cataloguing of many different metabolites involved in different pathways. Such an untargeted approach has been used to reveal differentiating metabolites between healthy individuals and those with Crohn's disease [129]. Metabolomics is also a powerful tool to decipher associations between the gut microbiome and xenobiotic compounds. However, the complex datasets generated from this technology require complex multivariate statistical analyses and careful interpretation of data,

especially since identification of masses is limited due to incomplete databases. Nevertheless, given their potential for clarifying complex interactions in microbial communities, their application in gut microbiome studies is promising.

2.5.2. From omics to causality: *in vitro* models

Integration of different culture-dependent, molecular and/or omics approaches allows us to pinpoint bacterial members of the GI community which are active, damaged, or responsive to a given ecological trigger (e.g. xenobiotic, disease pathology, diet, microorganisms, therapy, host immune system etc.) [229]. However, to validate the results and causally link the identified differences in the GI microbiota with the presence or absence of an environmental or host-related factor, experiments that allow for systematic manipulation of variables and experimental testing are essential. To this end, *in vivo*, *ex vivo*, *in silico* and *in vitro* models are critical. A comprehensive review covering a variety of these simulation approaches is available [230]. In the frame of this thesis, some *in vitro* models are illustrated below.

Static short-term batch fermentations are the simplest *in vitro* models in which a variety of cultures (e.g. specific strains, caecal, intestinal or faecal microbial communities from mammalian origin) are tested in single small vessels or test-tubes (e.g. for their ability to metabolize a specific substrate). The high throughput, easy set up and technical flexibility of these batch incubations make them highly suitable to test for interindividual differences and substrates of which only small amounts are available. However, changes in pH and redox potential combined with substrate depletion restrict the operational time and prevent the establishment of steady-state conditions [231,232]. Static batch fermenters are mostly used as a screening tool, but tend to oversimplify the actual complexity of the processes occurring in the GIT. Therefore, the subsequent application of controlled, continuous and dynamic models allows more in-depth study of the gut microbiome.

A major step forward was the design of a ***three-stage continuous fermentation model*** simulating the ascending, transverse and distal colon by the group of Gibson and Macfarlane in the 1980s [233]. The continuous flow better mimics *in vivo* conditions and the environmental parameters (e.g. pH, nutritional features, and fluid retention time) can be individually controlled per vessel [234]. Because inoculation with liquid faecal inocula may result in a rapid washout of less competitive bacteria, operational time of this type of model is limited to less than 4 weeks. To this end, immobilization of microbial cells within a porous polysaccharide matrix has improved stability during longer experiments. Moreover, members of the microbial community can develop in a planktonic (free-cell) or sessile (biofilm-associated) state [235].

The inclusion of the upper GIT in a continuous fermentation model led to the development of the ***Simulator of the Human Intestinal Microbial Ecosystem (SHIME)*** [236], which is a pioneering example of simulating the GIT as a whole. Five reactors are sequentially connected, with the first two reactors mimicking the stomach and small intestine following the fill-and-draw principle by adding a defined amount of nutritional medium, pancreatic enzymes and bile to the compartments. The three colon

compartments are continuously stirred reactors with constant volume and pH control mimicking distinct microbial fermentative processes that occur in the ascending, transverse and descending colons, respectively. A typical SHIME experiment consists of four stages: a stabilization period that allows the microbial community to adapt to the imposed *in vitro* conditions; a control period during which the system is operated under basal conditions; a treatment period during which the effect of a specific compound is tested; and a washout period to determine how long the induced changes can still be measured before return to the basal situation [237]. This validated dynamic model has been used in numerous studies to investigate the microbial conversion of specific food ingredients [238], to track the activity and stability of pre- and probiotics [239,240], to understand the pharmacokinetics of drugs [241] and to model the gut microbiota linked to gastrointestinal disorders [242]. Whereas most *in vitro* studies have targeted the luminal intestinal microbes, the contribution of the mucosal microbiota to immunological priming and intimate host-microbe interactions occurring at the mucosal barrier should not be neglected. This idea was brought into practice by incorporation of mucin-covered microcosms in the SHIME to build the so-called Mucosal-SHIME (M-SHIME). The latter model allows us to study the composition and functioning of specific mucosal microbes and their interaction with mixed luminal intestinal communities [242–244].

Other well-established models of the *in vitro* GIT include the dynamic computer-controlled ***TIM-1***, simulating the stomach and small intestine with peristaltic mixing and absorption of fermentation products and water, and the ***TIM-2*** mimicking the proximal colon of monogastric mammals [245,246].

Compared to *in vivo* models, *in vitro* models are not hampered by ethical considerations and limit discrepancies resulting from inter-individual variations that occur in *in vivo* animal models. Most systems are generally inexpensive to operate and easy to set up. They offer high flexibility, rapid throughput of samples, precise manipulation of environmental variables and dynamic sampling at different locations within the gut. Moreover, they provide a platform for mechanistic studies which include potentially radioactive, toxic and genotoxic substances. The immediate cause-and-effect read-outs can be assessed by downstream high-resolution molecular techniques.

An important limitation of virtually all GI models concerns the inherent difficulty to adequately simulate the contribution of the host. Consequently, the focus is mostly on the microbial side and biotic factors such as gut absorptive processes, secretions or defensive systems are not incorporated. The lack of feedback mechanisms from the host is one major drawback, though this actuates the *in vitro* living cell-based and microfluidics-based technologies. Conceptually, an ideal-experimental GI model should include host-derived cells, a representative microbiota sustainably growing under anaerobic or microaerophilic conditions, a mucus layer simulating the physical separation of host and microbial components, and a controlled physico-chemical simulation of the GI environment (**FIG 2.3**) [230].

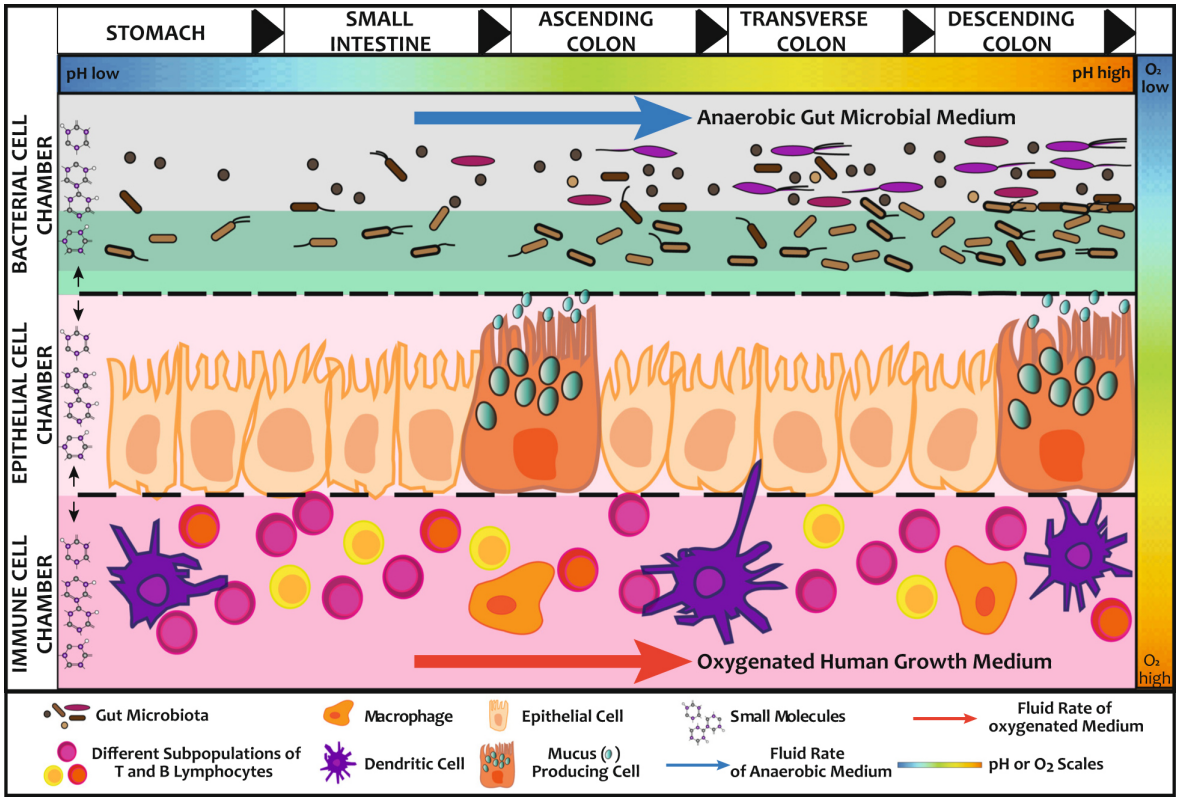

FIG 2.3 | Conceptualization of an idealized in vitro gastrointestinal experimental model. The individual compartments should ideally be connected in series and allow modulation of their respective factors including pH, fluid retention times, growth medium and physiological factors such as mucin compositions, which actively interact and alter the microbial communities (Figure reprinted with permission; © 2013 Fritz et al.; licensee BioMed Central Ltd., http://creativecommons.org/licenses/by/2.0/) [230]

3 CARNIVORANS AND THEIR GUT MICROBIOME: AN UNDEREXPLORED PARTNERSHIP

3.1. Implications of a strict carnivore heritage

Strict or obligate carnivores inherently rely on animal tissues to meet their specific and unique nutritional requirements. Among carnivorans, this biological necessity characterizes minks, otters, seals, walruses, sea lions as well as polar bears and all members of the *Felidae* including the **cheetah** [247]. Other obligate carnivorous mammals include for example dolphins, three-banded armadillos and tarsiers. What it means metabolically and nutritionally to be a strict carnivore has been most thoroughly studied in the domestic cat (*Felis catus*) and related *Felidae*. Some highlights of this research are summarized below.

Advantages of becoming carnivorous include the higher basal rates of metabolism, faster growth rates and higher fecundity as a result of the intake of meat that exceeds plant and arthropod foods in energy content [247]. The relative ease and speed of meat digestion is reflected in the anatomical adaptations of the feline GIT, with little or no compartmentalisation, limited length of the small intestine, short and rudimentary caecum and small colon. **Compared to cheetahs**, domestic cats possess slightly longer GIT relative to body size but this ratio is still 4:1 compared with 6:1 in omnivorous dogs and 27:1 in herbivorous sheep [19,248]. The natural carnivorous diet is high in protein with moderate amounts of fat and minimal amounts of carbohydrate, which mandates carnivores to use protein and fat as primary energy sources even when sources of protein in the diet are limiting. Two main reasons have been imputed to the particularly high dietary protein requirements of *Felidae*. The first is the higher basal requirement for nitrogen since felids lack the capacity to regulate hepatic enzyme activity in nitrogen metabolism, gluconeogenesis and lipogenesis, in response to altered dietary protein content. When fed a low-protein diet, most omnivores spare amino acids by reducing activities of enzymes involved in protein catabolism, whereas felids show little adaptation in the activities of the aminotransferases or urea cycle enzymes and retain high level of gluconeogenic activity. The second includes their unique requirement for taurine, arginine, cysteine and methionine due to deletion or downregulation of enzymes for synthesis of nutrients found abundantly in the diet and thus making their *de novo* synthesis redundant [248]. As with proteins, felids have also developed several unique adaptions to the metabolism of carbohydrates compared with omnivores or herbivores. These include the lack of salivary amylase and low activities of intestinal and pancreatic amylase in addition to low intestinal disaccharidase activity and the absence of glucokinase activity in the liver. As a result, felids have limited ability to rapidly minimize hyperglycemia from a large dietary glucose load [249].

Unique vitamin requirements are another result of a strictly meat-based diet. Carnivores have an absolute requirement for preformed vitamin A, which occurs naturally only in animal tissues, (especially within the viscera) because of the fact that they cannot convert carotenoids (plentiful in plants) to retinol (the active form of vitamin A). Insofar as a carnivorous diet has adequate amounts of vitamin D_3, pathways for synthesis would be unnecessary and thus carnivores lack also 7-dehydrocholesterol, which is required for vitamin D_3 synthesis. Felids also have a higher level of requirement for thiamine, pyridoxine, folate and niacin. The latter can be endogenously synthesized from tryptophan, but the efficiency of this conversion is rather low. Again, most of these requirements result from the fact that muscle tissue and/or organs are well supplied with these nutrients, making *de novo* synthesis a waste of energy [248,249]. As a consequence of the dietary specialization of strict carnivores such as the cheetah, it is likely that the nutritional content of diets fed to the captive population may differ from what their free-ranging counterparts catch in the wild. Moreover, zoo diets are also highly dependent on zoo management (storage and preparation), prey quality and availability.

3.2. Gut microbial diversity in carnivorans

For decades, the GI microbiota of carnivorans has attracted attention of investigators mainly due to its potential etiopathologic role in host health and disease. Because of this focus on a limited number of enteropathogenic species rather than on the entire GI community, very few microbiome data exist for carnivorans, let alone for obligate carnivorous carnivorans such as cheetahs. The fact that carnivorans don't rely primarily on their autochthonous GI microbiota for energy harvest and that they harbour a relatively simple GIT has left this type of studies off the stage, even though the digestive performance of all animals depends on both the genome of the host and the characteristics of the host's GI microbiome.

Recent work published by Ley *et al*. (2008) gave centre stage to diet specialization across vertebrates. This extensive study of the diversity of the microbiomes in 59 mammalian species (including two cheetahs) showed with a network-based analysis of bacterial 16S rRNA gene sequences that both phylogeny and diet and gut type have all driven the evolution of the gut microbiome [86]. Another highlight of the study was that bacterial diversity appeared to be lowest in carnivores, intermediate in omnivores and highest in herbivores. At large taxonomic scales diet appears to be a major driving factor, as gut microbiomes have evolved convergently in mammals sharing the same feeding habits [250]. Convergence driven by a herbivorous diet even occurs among vertebrates as exemplified by the higher numerical abundance of species from the Bacteroidia, Clostridia and Verrucomicrobia in the guts of herbivorous fish compared to other fish, showing close ties to gut microbiome composition in herbivorous mammals [251]. However, at shallower taxonomic scales (e.g. within bears), the microbial communities of conspecific hosts tend to be more similar to each other than to those of different host species sharing the diet type. This seems to indicate that sometimes diet has an overriding influence on the microbiome's structure, but sometimes common ancestry overrides the effects of diet. The

intertwining of diet and phylogeny is notably illustrated in the order Carnivora. For example, in spite of their herbivorous habits, giant pandas have simple guts with relatively reduced microbiomes that are more similar to those of their phylogenetically related carnivores, the omnivorous bears (*Ursidae*) [86]. Even upon comparison with red pandas, which are carnivorans adapted to the same highly specialized bamboo diet, the giant panda harbours different gut microbial communities and clustered closer to that of the black bears as analyzed by 454 pyrosequencing of the V1-V3 16S rRNA gene regions [252]. In contrast, neighbour-networks and beta-diversity plots generated from Illumina sequencing of the 16S rRNA gene do not group the gut microbiota of the sloth bear, an omnivorous bear specialized on ants and termites, with other bears. This suggests that the composition of their gut microbiota seems to have experienced an overall dietary-induced shift [253]. Similarly, the aardwolf is a strict myrmecophage (termite-eating specialist) closely related to hyaenas (*Hyaenidae*), but has diverged substantially from other members of the Carnivora and experienced a compositional shift of its gut microbiome to resemble other myrmecophages. Evolutionary trajectories followed by the gut microbiota thus differ greatly between carnivorans. In the case of the giant panda where a carnivore-like gut microbiome is retained, it is hypothesized that specific genes from the bacterial taxa are upregulated to facilitate cellulose and lignin degradation [254].

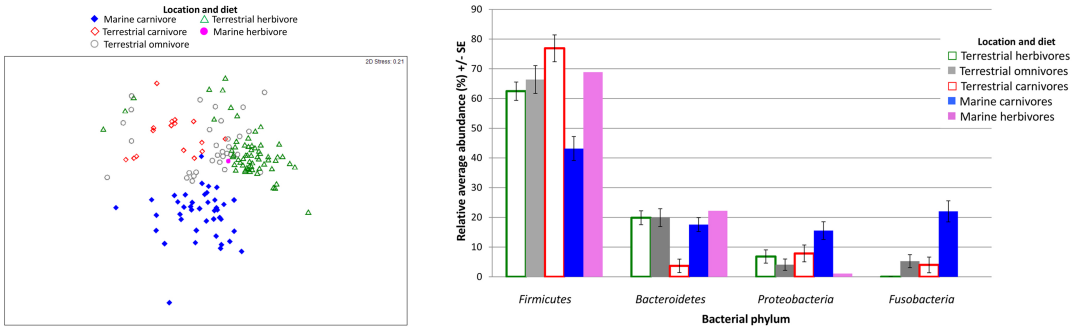

FIG 3.1 | Differences in diet and habitat result in divergent clustering of mammalian gut microbial communities and different abundances of dominant phyla. (Figure reprinted with permission; © 2013 Nelson *et al.*, http://creativecommons.org/licenses/by/4.0/) [255]

Compared to omnivores and herbivores, carnivores show signs of phylogenetic inertia and cluster together based on their microbiomes. In fact, large comparative studies included only two carnivores that did not belong to the Carnivora, *i.e.* Southern three-banded armadillo (order Xenartha) and short-beaked Echidna (order Monotremata) [86,250]. The included carnivorous members of the Carnivora were lions, cheetahs, polar bears, bushdogs and hyaenas, although these were limited in number (two or three individuals of each species). Moreover, marine carnivorous mammals were excluded from these comparative studies. Recently, Nelson and co-workers have compared the gut microbiomes from the previously mentioned datasets with those from several studies conducted in *Phocidae*, which include strict carnivorous seals. Data were generated from DGGE band sequencing, 16S rRNA gene clone libraries and 16S rRNA amplicon pyrosequencing [256,257]. They found that marine carnivores possess a richer gut bacterial community than their diet-equivalent terrestrial mammals. Separate clustering in an

nMDS ordination plot displaying similarity of the gut bacterial communities appears to be due, in part, to a considerably reduced abundance of Firmicutes and increased abundance of Fusobacteria compared to terrestrial carnivores (**FIG 3.1**) [255]. Moreover, upon comparison of different phylogenetic families within the order Carnivora, omnivorous dogs clustered more closely to carnivorous seals due to the greater abundance of Fusobacteria in their guts (**FIG 3.2**). This observation is supported by previously published data that noted a co-dominance of Firmicutes, Bacteroidetes and *Fusobacterium* in dogs and the phylogenetically related wolves as revealed by 16S rRNA gene clone libraries [258–260]. Importantly, this dominance of Fusobacteria in dogs appears to be a key difference between the canine and feline gut microbiome [261]. In contrast to marine carnivores, terrestrial obligate carnivores displayed a significantly reduced abundance of the phylum Bacteroidetes, which resulted in a predominance of members of the Firmicutes (**FIG 3.1**) [86,255]. However, the Firmicutes to Bacteroidetes ratio for sea lions seems to be more similar to terrestrial carnivores than to the one reported for other marine carnivorous mammals [262].

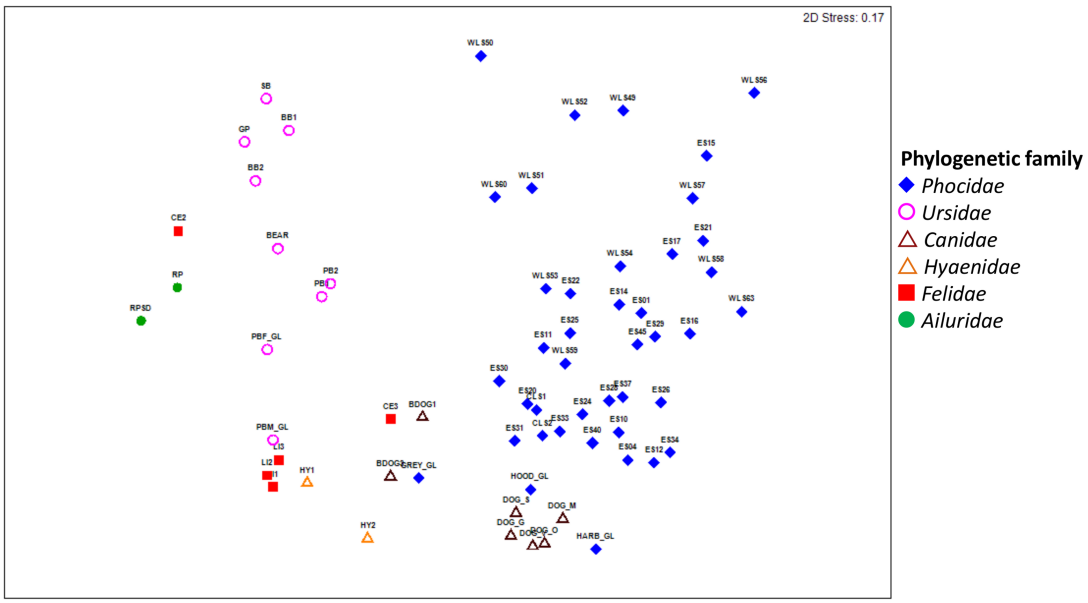

FIG 3.2 | Influence of phylogenetic family on the gut bacterial community of mammals belonging to the order Carnivora. Host species labels are as follows: (RP) red panda; (SB) spectacled bear; (GP) giant panda; (BB) black bear; (PB) polar bear; (BEAR) bear from Norway; (CE) cheetah; (LI) lion; (HY) spotted hyaena; (BDOG) bushdog; (DOG) domestic dog; (GREY) grey seal; (HOOD) hooded seal; (HARB) harbour seal; (ES) southern elephant seal; (L) leopard seal. Adapted from Nelson *et al.* 2013 [255]

A recent shotgun metagenomic study in a wild Iberian lynx reported a high abundance of the Bacteroidetes phylum [263], whereas an oligotyping study using Illumina MiSeq to sequence the V4 region of the 16S rRNA gene in **wild cheetahs** reported low abundances of Bacteroidetes comparable to the one found in most terrestrial carnivores [86,264]. At a finer taxonomic level, bacterial genera characteristic of the GI microbiota of terrestrial obligate carnivores (polar bears, bushdogs, hyaenas) include *Clostridium*, *Blautia*, *Coprococcus*, *Peptostreptococcus*, *Enterococcus* and *Lactobacillus* [255]. In addition, high abundances of *Clostridium* cluster I and XI in faecal samples of grizzly bears were

positively correlated with diet protein content, which is typically high in a carnivorous diet [265]. Other studies in polar bears, snow leopards, and **cheetahs** confirmed the higher proportion of the genera *Blautia* and *Clostridium* [264,266,267]. *Clostridium* was also prominent in omnivorous grizzly bears and herbivorous pandas, next to high proportions of *Enterobacteriaceae* [80,268,269]. Although the use of different methodologies for sampling, sample processing and taxonomic allocation as well as environmental and animal-related factors might partially explain the different proportions of bacterial groups, the carnivore-like microbiota seems to be characterized by the enrichment of lineages from the phylum Firmicutes, especially *Clostridium*, *Blautia*, *Coprococcus* and *Enterococcus*, and a dearth of Bacteroidetes, *Ruminococcaceae* and *Bifidobacteriaceae* compared to other mammalian species. In addition, Muegge *et al.* (2011) also identified differences among the functional configurations of microbiomes, with mostly enzymes involved in amino acid metabolism distinguishing carnivorous and herbivorous species. Microbiomes from herbivores were enriched in enzymes that map to biosynthetic reactions, whereas the amino acid degradation pathways were significantly increased in carnivores (e.g. catabolic breakdown of arginine, glutamine, glutamate and proline) [250].

3.3. Feline gut microbial diversity

Although *Felidae* are a large family of well-known terrestrial obligate carnivores, only a few of its members have been included in comparative microbiome studies (*i.e.* three lions, **two cheetahs**, one lynx and one snow leopard). This is in contrast to their domestic counterpart, of which the gut microbiome has been extensively studied and reviewed during the last decade [261,270–274]. Several approaches have been used to determine the phylogeny and functional capacity of the gut microbiome of domestic cats. Culture-based studies have mostly revealed the presence of *Clostridium* spp., *Enterococcus* spp., *Streptococcus* spp., *Fusobacterium* spp., *Eubacterium* spp. and *Bacteroides* spp., and also reported a higher proportion of obligate anaerobic bacteria in the small intestines (10^5-10^8 CFU/ml) compared with humans and dogs [275–279]. Studies using FISH reported highest counts for the *Atopobium* group, *Clostridium* cluster I, *Lactobacillus* spp., *Enterococcus* spp., *Bacteroides* spp. and *Bifidobacterium* spp., though the latter varied to a greater extent between both studies (11% to 30% of total counts) [280,281]. Ritchie *et al.* (2008) used a 16S rRNA gene-based approach to identify the most predominant bacterial phyla: Firmicutes (68%), Proteobacteria (14%), Bacteroidetes (10%), Fusobacteria (5%) and Actinobacteria (4%). The majority of the identified clones fell within the order Clostridiales, followed by Lactobacillales in the jejunum and Bacteroidales in the colon [282]. Based on the use of another molecular target, *i.e.* the *cpn60* gene, members of the Firmicutes (41-72%) were found to be predominant, followed by Actinobacteria, Bacteroidetes and Proteobacteria [283]. Using 16S rRNA gene 454 pyrosequencing methods, the reported most abundant faecal bacterial phylum was Firmicutes (92-95%), followed by Actinobacteria, with less than 1% present being Proteobacteria, Bacteroidetes and Fusobacteria [284]. This was confirmed by another study using massive parallel 16S rRNA gene pyrosequencing which reported Firmicutes (92%) as the most abundant phylum, followed by

Actinobacteria (7.3%), Bacteroidetes (0.45%) and Fusobacteria (0.04%). Within the class Clostridia, *Ruminococcus* and *Clostridium* clusters XIVa and XI prevailed [285]. In contrast, a recent 454 pyrosequencing study described co-dominance between Firmicutes (36-50%) and Bacteroidetes (24-36%) [286]. Another metagenomic study on pooled faecal samples from five cats revealed that Bacteroidetes was the most predominant phylum (68%), followed by Firmicutes (13%), Proteobacteria (6%), Actinobacteria (1.2%) and Fusobacteria (0.7%) [287]. Next to the influence of host-related (e.g. age, genetic background, health status etc.) and environmental factors (e.g. housing, diet etc.), some of the reported differences in proportional abundance of bacterial groups may also stem from differences in methodological sensitivity and specificity. This also complicates comparison of proportional abundances of bacterial groups between different members of the *Felidae*. Lions and **cheetahs** seem to harbour high abundances of Firmicutes (76-95%), with *Clostridium* being most prevalent [86,264], whereas lynx tend to have a co-dominance of Firmicutes (43%) and Bacteroidetes (39%) [263]. However, the number of samples and animals used in these comparisons is very limited. This again highlights the need for a comprehensive study including a representative number of different feline species to determine the signatures of a feline microbiome and to compare these with microbiomes from other obligate carnivores. In this context, the review of Minamoto and co-workers on the predominant GI microbiota of domestic cats provides a valuable starting point to initiate further work (**FIG 3.3**) [272]. Next to insights from phylogeny, some metagenomic studies have also assessed the functional capacity of the faecal microbiome of healthy adult cats. It has been reported that microbial carbohydrate and protein metabolism accounted for approximately 13-14% and 8-9% of the feline metagenome, respectively [286,287]. These results were comparable to those reported for the canine metagenome [288]. Despite the obligate carnivorous nature of cats, their GI microbiome appears to contain the functional machinery similar to that of other carnivorans. Many more in-depth studies are needed to test this functional capacity and plasticity, and how it compares with microbiomes of other host species in terms of substrate specificity, enzyme kinetics, metabolite production and impact on host health.

Prior to the onset of this PhD, specific insights in the **gut microbial diversity of cheetahs** were as good as non-existent. In an early study published in 1993, bacterial cultures from captive cheetah rectal swabs were compared to those from domestic cats. Of particular note was the absence of *Bacteroides* and *Lactobacillus* spp. in the cheetah isolates compared to their presence (46.7 and 36.7%, respectively) in rectal isolates from domestic cats [289]. Recent work published by Ley *et al*. (2008) provided the first 16S rRNA gene-based insight into the cheetah's gut microbiome by including two captive cheetahs in a network-based analysis of bacterial 16S rRNA gene sequences generated by 16S rRNA gene clone libraries of 59 mammalian species. Results showed significant differences in proportions of phyla present in both cheetahs. Firmicutes was predominant (50 vs 94%), followed by Actinobacteria (24% vs 0%), Proteobacteria (24% vs 2%), and a minority of Fusobacteria (0% vs 3%) and Bacteroidetes (2% vs 0%). At family level, *Clostridium* clusters XIVa and XI, *Peptostreptococcaceae* and *Lachnospiraceae* prevailed [86].

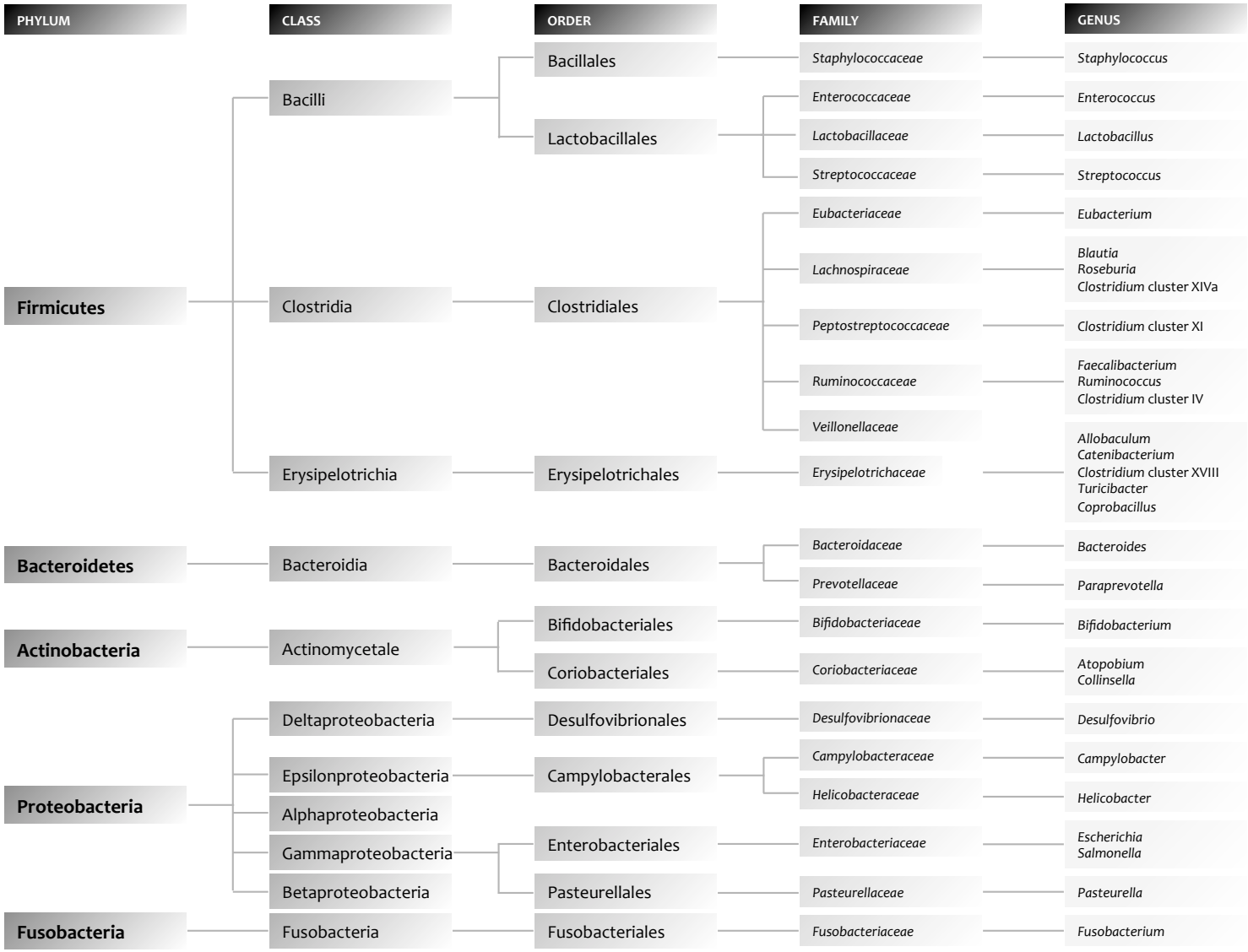

FIG 3.3 | Taxonomic lineages of the predominant bacterial groups identified in the feline gastrointestinal tract using molecular methods. [272]

3.4. Microbial shaping of feline carnivorans

The initial metagenomic projects dedicated to domestic cats and phylogenetically related species have provided a first glimpse of the complex microbiome residing in the simple gut of carnivores and the mammalian clade Carnivora. Similar to humans and other mammals, this has led to an increase in studies focusing on microbiome-host metabolism interactions. The research community has identified two main targets in this context. The ***nutritional manipulation of the GI microbiota*** is of key interest to the petfood industry, whereas the ***cause-and-effect relationship between microbiota and host health*** conditions is driven by the interest of the veterinary sector.

3.4.1. The feline microbiome involved in chronic enteropathies

Similar to what is observed in humans and dogs, quantitative and qualitative alterations of the intestinal microbiota are associated with the etiopathogenesis of several diseases in domestic cats. For example, diarrhoea is one of the most common reasons for cats to be presented to a veterinarian and has been associated with several potential enteropathogens, such as *Clostridium perfringens*, *Clostridium difficile* and *Salmonella* spp. [272]. However, because these same species have also been commonly isolated from healthy animals, such observations do not necessarily prove causation [290]. Recently, significant differences in the faecal microbiomes between healthy cats and cats with diarrhoea were identified for the first time using next-generation sequencing [291]. An overall decrease in species richness was observed in diarrheic cats, similar to previous observations in dogs with GI disease [292]. The order Burkholderiales, the family *Enterobacteriaceae*, and the genera *Clostridium*, *Collinsella* and *Streptococcus* were significantly increased in diarrheic cats, whereas important producers of SCFAs such as members from *Clostridium* clusters XIV and IV were decreased. The latter observation is consistent with data observed in dogs. An increase in *Clostridium perfringens* has been considered as a marker of intestinal dysbiosis in dogs [272,291,293], but qPCR-based quantification did not reveal a significant increase of this species in diarrheic cats. On the functional level, the dysbiosis in diarrheic cats was accompanied by changes in bacterial gene categories suggesting a disturbed amino acid metabolism and a decreased abundance of genes responsible for vitamin metabolism [291].

Another type of chronic enteropathy in domestic cats associated with a disrupted microbial community is inflammatory bowel disease (IBD). In one study using FISH analysis, total bacteria, *Bifidobacterium* spp. and *Bacteroides* spp. counts were significantly lower in cats with IBD whereas *Desulfovibrio* spp. counts were higher [281]. However, this was not reported in another study targeting the same bacterial groups with FISH [280]. An increased count of mucosa-associated *Enterobacteriaceae* was also reported in duodenal biopsies of cats with IBD [294]. However, to date high-throughput sequencing has not been used for the characterization of feline IBD, in contrast to canine IBD. Dogs with IBD have been shown to have lower abundance of Bacteroidetes, Fusobacteria, *Prevotellaceae* and Clostridiales, but higher counts for Proteobacteria when compared with healthy dogs [295].

Although the importance of maintaining a balanced intestinal microbial ecosystem in carnivores is acknowledged, the cause-and-effect relationships are still unclear. Moreover, because faecal 16S rRNA gene-based studies provide information only about the taxonomic composition of healthy and diseased microbiomes, microbial transcriptome and metabolome data along with more host physiological data are needed to increase our understanding in this regard.

3.4.2. The feline microbiome as nutritional target

Commercial petfoods are highly variable with regard to format, macronutrient composition, fibre content and source and inclusion of functional ingredients. Recent studies have investigated the effects of diet on the feline gut microbiota using high-throughput sequencing techniques. Given their carnivorous nature, it has been hypothesized that the protein to carbohydrate ratio is important for feline health. As such, the effect of dietary protein to carbohydrate ratio on the faecal microbiota of growing kittens was evaluated using 16S rRNA gene pyrosequencing. A greater abundance of *Clostridium*, *Blautia*, *Faecalibacterium*, *Ruminococcus* and *Eubacterium* as well as a reduced faecal *Bifidobacterium* number were reported when kittens were fed a high-protein and low-carbohydrate diet. The authors suggested that the latter could have been due to the negative effects of metabolites resulting from an increased flow of undigested protein to the colon [296]. Using the same faecal samples, Illumina shotgun sequencing was used to explore the functional capacity and revealed strong diet-related differences in amino acid biosynthesis [297]. Another study focused on the different impact of commercial dry (crude protein=33%; crude fat=11% on dry matter basis) and wet (crude protein=42%; crude fat=42% on dry matter basis) food on faecal microbial populations in cats using 16S rRNA gene amplicon-based 454 pyrosequencing [298]. Cats fed the dry diet showed a higher abundance of Actinobacteria and lower proportion of Proteobacteria and Fusobacteria. In addition, the dry diet increased the number of *Lactobacillus* spp. but decreased *Bacteroides* and *Blautia*. However, these shifts could not be attributed to specific nutrients of the diets, only to the diets as a whole.

Another emerging topic has been the impact of dietary fibre, prebiotics and probiotics on the GI microbiota [51,299]. Prebiotics are selectively fermented ingredients that cause specific changes in the composition and/or activity of the GI microbiota to benefit the host, whereas probiotics can be defined as live microorganisms that, if consumed in adequate amounts, could provide a health benefit to the host [300]. For example, cats fed a diet supplemented with fructooligosaccharides (FOS; 4% of the diet) had increased bifidobacterial concentrations while counts of *Escherichia coli* decreased. Then again, the inclusion of pectin (4% of the diet) increased proportions of Firmicutes and total bacteria. The study in question used shotgun 454-pyrosequencing and also reported an increased abundance of genes associated with amino acid metabolism with the FOS diet, while the pectin diet increased the genes associated with N metabolism [286]. Another example includes the evaluation of a multi-species synbiotic supplement on the faecal microbiota of healthy cats by 16S rRNA gene amplicon-based 454-pryosequencing. No changes in the major phyla nor gastrointestinal function or immune markers were observed, and also no adverse gastrointestinal effects were recorded [284]. Recently, two commercial

therapeutic diets were evaluated in cats with chronic diarrhoea. Due to the lack of a healthy control group and because the tested diets contained many different ingredients, no specific cause-and-effect relationships were elucidated, although strong correlations between microbiota and faecal score were observed [301].

Overall, many of the aforementioned studies seem to demonstrate that diet may modulate the composition of the GI microbiota and its functional pathways. However, most of them have not reported enough data to establish cause-and-effect metabolic relationships between bacterial groups and the host. Identifying the activity and responsiveness of the GI microbiota and mechanisms by which they affect feline host GI heath and metabolic diseases warrants a multi-disciplinary approach. This should involve *in vivo* and *in vitro* studies to complement insights from DNA sequencing technologies with functional omics techniques and in-depth nutritional analyses. The combined information will then be useful to design dietary or environmental strategies that may alter the GI microbiota, on a phylogenetic and/or functional level, resulting in a beneficial outcome for the feline host.

Although such studies in domestic cats are within reach, the impact of dietary modulation on gut microbial composition and functioning in other (feline) carnivores and consequences for their (gastrointestinal) health remain largely an unexplored area. Moreover, bacterial target species for dietary modulation are poorly known in those carnivorous hosts. In particular, endangered feline carnivores such as cheetahs might benefit from a multi-disciplinary approach to assess and assure their health status in captivity. To this end, characterization of the gut microbiota, which is an important intermediate between diet and host, can provide novel insights to steer dietary strategies. This PhD work focused on unraveling the cheetah's gut microbial composition, given its particular relevance in gastrointestinal diseases among captive cheetahs and its potential role in converting animal fibre substrates from prey diets into host-beneficial metabolites [49]. Moreover, the study of gut microbiomes in feline carnivores other than domestic cats can provide a framework to (re-)evaluate to what extent the domestic cat can serve as a model for other feline species.

SUPPLEMENTARY DATA

TABLE S1 | Categories of diversity measurements [157].

	Measurement of diversity within a single community (α diversity)	Measurement of diversity shared among communities (β diversity)
Presence/absence of taxa considered	**Qualitative α diversity** · Richness **Species-based:** · Chao 1 · ACE · Rarefaction **Divergence-based:** · Phylogenetic Diversity (PD)	**Qualitative β diversity** **Species-based:** · Sörensen index · Jaccard index **Divergence-based:** · Unweighted UniFrac · Taxonomic Similarity (Δ_S)
Additionally accounts for the number of times that each taxon was observed	**Quantitative α diversity** · Richness and/or evenness **Species-based:** · Shannon's index · Simpson's index **Divergence-based:** · Theta	**Quantitative β diversity** **Species-based:** · Sörensen quantitative index · Morisita-Horn measure **Divergence-based:** · Weighted UniFrac · F_{ST} · DPCoA

PART III | OBJECTIVES

While there is increasing evidence for the pivotal role of the GI microbiota in mammalian host homeostasis and susceptibility to disease, very little data on the microbiome of obligate carnivores exist. The initial sequencing projects in domestic cats have merely provided a glimpse of the complex microbiome residing in the simple gut of this specific group of carnivores. The intestinal microbiota is dependent upon the enzymatically undigested portion of the carnivorous diet as the main source of substrates for its metabolism. This undigested portion is markedly different between domestic cats, mostly fed commercial petfoods, and large exotic felids in captivity, which consume raw vitamin- and mineral-supplemented muscle meat or whole prey including different types of non-digestible tissues (e.g. fur, cartilage, skin, bone). These animal-derived components have been postulated to act as animal fibre since they beneficially changed fermentation profiles in captive cheetahs [49]. This hypothesis disclosed an important aspect largely ignored when assessing feeding strategies in exotic felids: the impact of diet type on GI homeostasis through altering the intestinal microbiota ecology and its associated fermentation processes. However, in order to predict the outcome of health promotion in exotic felids through nutritional modulation, it is crucial to first obtain comprehensive insights in their intestinal microbial ecosystem. Furthermore, the gathered knowledge can then be used in specific *in vitro* gut simulation models to evaluate the effect of different types of nutritional modulation. However, optimized *in vitro* models simulating the feline GI tract are currently not available.

This work focuses on the captive cheetah population which suffers from a high incidence of GI and metabolic diseases and low breeding rates. Given the fact that the cheetah population in zoos is currently not self-sustaining but important to the survival and conservation of this vulnerable to critically endangered species, optimal health conditions in captivity are warranted. Since nutrition underpins the majority of health parameters, proper feeding strategies will benefit the population. In this context, fundamental microbiological insights will provide a framework to further evaluate the effect of different diet types on the cheetah's GI health and general well-being in captivity.

To this end, two main objectives are envisaged:

(i) to explore the diversity and dynamics of the predominant members of the captive cheetah's intestinal microbiota leading to the first ever taxonomic benchmark using culture-independent techniques

(ii) to evaluate the application of an *in vitro* feline gut simulation model for studying nutritional modulation of the cheetah's intestinal microbiota and its associated fermentation processes

The ***first objective*** was subdivided into four specific aims.

- Construction of a phylogenetic framework for identification of the cheetah's GI bacterial diversity based on two animals housed in a Belgian zoo using 16S rRNA gene clone libraries (**Chapter 4**)
- Monitoring of the long-term temporal stability of cheetah GI microbiota in five animals from two different zoos using DGGE community fingerprinting and a combination of real-time PCR assays (**Chapter 5**)
- Exploration of the added value of Illumina high-throughput sequencing to study cheetah GI microbiota diversity and dynamics in samples previously analysed with conventional molecular techniques (**Chapter 6**)
- Community profiling of GI microbiota in captive and free-ranging conspecifics of the cheetah (**Chapter 7**)

The ***second objective***, discussed in **Chapter 8**, was subdivided into three specific aims.

- Adaptation of the *in vitro* Simulator of the Human Intestinal Microbial Ecosystem (SHIME) model into a dynamic system simulating the feline carnivorous gastrointestinal tract
- Evaluation of the metabolic and compositional steady-state conditions in the adapted *in vitro* feline gut simulation model inoculated with cheetah faecal inoculum using a combination of conventional metabolic analyses, DGGE community fingerprinting and a high-throughput untargeted metabolomics approach
- Exploration of the potential of the adapted feline model to investigate metabolic and compositional effects of dietary supplementation using (hydrolyzed) collagen as a test case

PART IV | EXPERIMENTAL WORK

Redrafted from:

Becker AAMJ, Hesta M, Hollants J, Janssens GPJ, Huys G (2014) Phylogenetic analysis of faecal microbiota from captive cheetahs reveals underrepresentation of Bacteroidetes and Bifidobacteriaceae. BMC Microbiology 14:43

4 PHYLOGENETIC ANALYSIS OF FAECAL MICROBIOTA FROM CAPTIVE CHEETAHS REVEALS UNDER-REPRESENTATION OF BACTEROIDETES AND *BIFIDOBACTERIACEAE*

Imbalanced feeding regimes may initiate gastrointestinal and metabolic diseases in endangered felids kept in captivity such as cheetahs. Given the crucial role of the host's intestinal microbiota in feed fermentation and health maintenance, a better understanding of the cheetah's intestinal ecosystem is essential for improvement of current feeding strategies. We determined the phylogenetic diversity of the faecal microbiota of the only two cheetahs housed in an EAZA associated zoo in Flanders, Belgium, to gain first insights in the relative distribution, identity and potential role of the major community members.

Taxonomic analysis of 16S rRNA gene clone libraries (702 clones) revealed a microbiota dominated by Firmicutes (94.7%), followed by a minority of Actinobacteria (4.3%), Proteobacteria (0.4%) and Fusobacteria (0.6%). In the Firmicutes, the majority of the phylotypes within the Clostridiales were assigned to *Clostridium* clusters XIVa (43%), XI (38%) and I (13%). Members of the Bacteroidetes phylum and *Bifidobacteriaceae*, two groups that can positively contribute in maintaining intestinal homeostasis, were absent in the clone libraries and detected in only marginal to low levels in real-time PCR analyses.

This marked underrepresentation is in contrast to data previously reported in domestic cats where Bacteroidetes and *Bifidobacteriaceae* are common residents of the faecal microbiota. Next to methodological differences, these findings may also reflect the apparent differences in dietary habits of both felid species. Thus, our results question the role of the domestic cat as the best available model for nutritional intervention studies in endangered exotic felids.

4.1. Background

In the broad scope of wildlife conservation with the aim to protect animal species from extinction, researchers and zoo managers face significant challenges in the conservation of threatened and endangered species. In zoo animal husbandry, nutrition is one of the most critical components [302]. Feeding mismanagement may give rise to suboptimal health, low breeding performance and a higher incidence of gastrointestinal and metabolic diseases [32,34,303]. In this context, well-balanced diets represent an important route for prevention or therapeutic intervention [284,304].

Due to diet-induced evolutionary adaptations, cats have developed a strictly carnivorous lifestyle with unique nutrient requirements [305]. Extrapolations of the dietary profile of the domestic cat to wild felids in captivity have been made [306,307] but are highly debatable since great differences exist in regards to their anatomical, behavioral and nutritional characteristics. Domestic cats are subjected to frequent feeding portions of carbohydrate-rich extruded kibble diets [308]. In contrast, captive exotic felids are usually fed once a day commercially available raw meat, sometimes supplemented with a vitamin and mineral premix, or whole carcasses [309]. The latter comes with variable amounts of indigestible animal tissues, such as raw bones, tendons, cartilage, skin, hair or feather. The undigested portion of the diet provides the main source of fermentable substrates for the intestinal microbiota, which form the main go-between in the translation of nutritional properties of the diet to health benefits for the host [310]. Comparison of mammalian gut microbiotas has shown that diet is, next to gut physiology, a major regulator of faecal microbiota composition [311].

In domestic cats, taxonomic and functional studies of the intestinal microbial communities have shown that different sources of dietary fibre (*i.e.*, cellulose, pectin, fructooligosaccharide) modified the composition of bacterial phyla in the faeces. For instance, cats fed a diet containing 4% pectin were found to display a higher percentage of Firmicutes and Spirochaetes than cats fed a diet containing 4% cellulose [312]. In the same study, dietary fructooligosaccharides increased the percentage of Actinobacteria. Conversely, high-protein diets induced a microbial shift towards decreased *E. coli*, *Bifidobacterium* and *Lactobacillus* populations [313,314]. In captive exotic felids, however, information on the composition and dietary modulation of the intestinal microbiota remains scarce [306].

Recent *in vivo* and *in vitro* studies in one of the most endangered exotic felid species, the cheetah (*Acinonyx jubatus*), point towards a significant role for microbial degradation of undigested animal tissues in the host's metabolic homeostasis [49,52]. However, because the number of captive animals available for well-documented faecal sample collection is extremely limited and because the composition and the functional capacity of the cheetah microbiota is virtually unknown, it has not been possible to link these observations to specific bacterial shifts or adaptations in the intestinal ecosystem. In addition, direct extrapolation of microbiological insights obtained for the domestic cat is not a valid approach given its adaptation to commercial diets. To start bridging the knowledge gap between the design of nutritional intervention strategies and the prediction of potential health benefits, this study aimed to inventorize the predominant

faecal microbiota of the only two captive cheetahs held in a zoo in Flanders (Belgium) associated with the European Association of Zoos and Aquaria (EAZA). Compositional analysis of 16S rRNA gene clone libraries was used for classification of the obtained phylotypes at phylum and family level, leading to the identification of the major bacterial groups that compose the cheetah's intestinal ecosystem.

4.2. Material & Methods

Ethics statement

This study was conducted non-invasively, without animal handling or any change in daily management and housing conditions of the animals. The cheetahs are housed according to the Minimum Standards for the Accommodation and Care of Animals in Zoos and Aquaria and the EAZA Code of Practice. Permission for faecal sampling was obtained from the staff veterinarians and mammal curators. Ghent University Animal Ethics Committee approval and any additional permits were not further needed.

Sample collection

Fresh faecal samples (200 gram) were collected in 2011 from the two adult male cheetahs (B1 and B2; both 10 years old) housed at Zooparc Planckendael (Flanders, Belgium), a full member of EAZA (http://www.eaza.net/membership). The animals shared indoor and outdoor housing and were fed their regular zoo diet *i.e.* chunked boneless horsemeat (2 kg/day/animal) topdressed with a vitamin and mineral premix (Carnicon®; Aveve, Leuven, Belgium) randomly interspersed with unsupplemented whole rabbits. No medical or health problems were reported or apparent on remote examination, and both cheetahs were treated prophylactically for internal parasites (Horseminth®; Pfizer, Brussels, Belgium). Faecal samples were immediately collected upon defaecation into plastic tubes, transported on dry ice and stored at -80°C until further analysis.

DNA extraction

Prior to DNA extraction, 25 grams (wet weight) of each thawed faecal sample was placed separately in sterile stomacher bags and homogenised in 225 ml peptone-buffered saline (PBS) (0.1% [wt/vol] bacteriological peptone [L37; Oxoid, Basingstoke, United Kingdom], 0.85% [wt/vol] NaCl [106404; Merck, Darmstadt, Germany]). The sludgy homogenate was filtered on a Büchner funnel to discard large particles such as hair and bones, and subsequently divided into 1.5 ml aliquots which were stored at -80°C.

The protocol of Pitcher et al. (1989) [315] was used in a modified version [210] to extract total bacterial DNA from the faecal samples. DNA size and integrity were assessed on 1% agarose electrophoresis gels stained with ethidium bromide. DNA concentration and purity were determined by spectrophotometric measurement at 234, 260 and 280 nm. DNA extracts were finally diluted ten times with TE buffer (1 mM EDTA [324503; Merck, Darmstadt, Germany], 10 mM Tris-HCl [648317; Merck, Darmstadt, Germany]) and stored at -20°C.

Real-time PCR

Quantitative PCR amplification and detection were performed using the Roche Light Cycler 480 machine with the Roche Light Cycler 480 SYBR Green I Master kit. Each PCR reaction included 40 ng DNA. Specific primers were used for Bacteroidetes (Bact934F [5' GGARCATGTGGTTTAATTCGATGAT 3'] and Bact1060R [5' AGCTGACGACAACCATGCAG 3']) and Firmicutes (Firm934F [5' GGAGYATGTGGTTTAATTCGAAGCA 3'] and Firm 1060R [5' AGCTGACGACAACCATGCAC 3']), along with universal primers for total bacteria (Eub338F [5' ACTCCTACGGGAGGCAGCAG 3'] and Eub518R [5' ATTACCGCGGCTGCTGG 3']) as previously described [316]. Samples were incubated at 95°C for 5 min and subsequently amplified during 45 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 1 s. The relative amount of Firmicutes and Bacteroidetes 16S rRNA in each sample was normalized to the total amount of faecal bacteria amplified with 16S rRNA gene-based universal primers [194,317]. *Bifidobacteriaceae* were quantified using *Bifidobacterium*-specific primers g-Bifid-F (5' CTCCTGGAAACGGGTGG 3') and g-Bifid-R (5' GGTGTTCTTCCCGATATCTACA 3') [318].

The ability of primers Bact934F and Bact1060R to detect members of the Bacteroidetes phylum in cheetah faeces was evaluated in a spiking experiment. For that purpose, *Bacteroides fragilis* DSM 1396, *Bacteroides uniformis* DSM 6597 and *Bacteroides distansonius* DSM 20701 were cultured anaerobically at 37°C for 48h on Reinforced Clostridial Medium (RCM) (M37; Oxoid, Basingstoke, United Kingdom). Inocula were prepared from harvested colonies and enumerated by plating serial 10-fold dilutions. Similarly, RCM counts were determined for faecal homogenates of B1 and B2. These homogenates were spiked with an equivalent mixture of the three *Bacteroides* strains at 1%, 10% and 50% of the total RCM count. Spiked samples were subjected to DNA-extraction and real-time PCR as described above.

Community PCR

Template DNA obtained from cheetahs B1 and B2 was subjected to 16S rRNA gene amplification using the conserved primers pA (5′ AGA GTT TGA TCC TGG CTC AG 3′) and pH (5′ AAG GAG GTG ATC CAG CCG CA 3′) which flank respectively the extreme 5' and 3' part of the 16S rRNA gene, thus allowing amplification of the entire gene [319]. Each reaction mixture (50 µl) contained 5 µl 10x PCR buffer (100 mM Tris-Hcl, pH 8.3 [at 25°C]; 500 mM KCl; 15 mM $MgCl_2$; 0.01% [wt/vol] gelatin [GeneAmp®; Applied Biosystems, USA]), 1 µl 25 mM $MgCl_2$, 5 µl 2 mM dNTPs (GeneAmp®; Applied Biosystems, USA), 0.04 µl 10 µg/µl bovine serum albumin, 1.25 µl 1 U/µl AmpliTaq® (Applied Biosystems, USA), 2.5 µl of each 10 µM primer, 4 µl template DNA and milliQ water to 50 µl. The samples were amplified in the VeritiTM Dx 96-Well Thermal Cycler (Applied Biosystems, USA), using the following PCR programme: initial denaturation at 94°C for 5 min followed by 18 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, with a final extension of 72°C for 10 min. Negative (milliQ water as template) and positive controls (Marinobacter sp. strain T278 [R-39409]) were included in parallel. Amplicons were checked on a 1% agarose gel under UV illumination after ethidium bromide staining of the gel, and subsequently purified with the QIAquick® PCR purification kit (Qiagen, Germany).

Cloning of bacterial 16S rRNA gene amplicons

For both cheetahs B1 and B2, a clone library was prepared. Purified 16S rRNA gene amplicons were ligated into the pGEM®-T Vector System (Promega Benelux, The Netherlands) and transformed into competent *E. coli* cells according to the manufacturer's instructions. White clones were amplified using the primer pair T7 (5' AAT ACG ACT CAC TAT AGG 3') and Sp6 (5' ATT TAG GTG ACA CTA TAG 3') to determine the size of the inserts.

Sequencing and sequence processing

The diversity of the clone libraries was examined via short fragment sequencing on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, USA) by means of the Big Dye® XTerminatorTM v.3.1. Cycle Sequencing and Purification Kit (Applied Biosystems, USA) according to the protocol of the supplier. The sequencing primer used was BKL1 [320]. For each sample, clones were sequenced, assembled in BioNumerics (Applied Maths, Sint-Martens-Latem, Belgium) and edited to exclude the primer binding sites. Chimeras were detected using Bellerophon [321] and B2C2 [322], and excluded for further analysis.

Phylogenetic analyses

Chimera-free sequences were aligned using ClustalW in MEGA 5.0 [323] and corrected by manual inspection. Homology searches were performed via BLAST [324], and taxonomic classification of the 16S rRNA transcripts was obtained by comparison against The Ribosomal Database Project-II (RDP) [325]. Only annotations with a bootstrap value over 0.8 were considered as well identified phylogenetic levels, leaving successive levels as unclassified. Groups of sequences with ≤ 3% sequence divergence (≥97% similarity) were defined as an operational taxonomic unit (OTU) or phylotype. Rarefaction curves were determined for different clone library sizes and Good's coverage index [326] was calculated as 1-(n/N) x 100, where n is the number of singleton phylotypes and N is the total number of sequences in the sample. From each OTU at the 97% cut off, a representative clone was selected along with its nearest type strain from the RDP database. A similarity-matrix was calculated using the Maximum Composite Likelihood parameter and data were visualized in a neighbour-joining phylogenetic tree constructed in MEGA 5.0. Reliability of the tree was evaluated based on 1000 bootstrap replicates.

Availability of supporting data

The data set supporting the results of this article is available in the GenBank repository, accession numbers KF909375 – KF910074.

4.3. Results

4.3.1. Distribution of OTUs in 16S rRNA gene clone libraries

Two clone libraries (CL-B1 and CL-B2) were created using the full-length 16S rRNA gene amplicons from samples B1 and B2. Although most of the DNA inserts corresponded to the expected full-length amplification products, some clones contained short fragments probably due to internal restriction sites. A selection of 384 clones per library was sequenced with primer BKL1, resulting in 352 and 350 quality-checked sequences of 400 to 450 bp length from the 5' end for libraries CL-B1 and CL-B2, respectively. With a 97% sequence identity criterion, 29 OTUs were obtained for CL-B1 and 37 OTUs for CL-B2. The coverage of the clone libraries was 98.6% and 97.7%, respectively, according to Good's formula [326]. Among the 66 OTUs, only 18 were found to be common to both libraries. Together, these common OTUs represented 298 sequences (84.7%) in CL-B1 and 317 sequences (90.6%) in CL-B2. Among the remaining OTUs, 11 OTUs were unique to clone library B1 and 19 to clone library B2. Rarefaction curves were obtained by plotting the number of phylotypes observed from both samples against the number of clones sequenced. The decrease in the rate of phylotype detection indicates that the majority of the predominant bacterial diversity in these samples was covered by clone library analysis (see **FIG S4.1**).

4.3.2. Taxonomic composition of 16S rRNA gene clone libraries at phylum and family level

Firmicutes was by far the most abundant bacterial phylum representing 96.6% and 92.9% of all sequences in CL-B1 and CL-B2, respectively. Three other bacterial phyla formed a minority in the phylogenetic spectrum, *i.e.* Actinobacteria (3.1% in CL-B1; 5.4% in CL-B2), Proteobacteria (0.3% in CL-B1; 0.6% in CL-B2) and Fusobacteria (1.1% in CL-B2). Surprisingly, none of the sequences was assigned to the Bacteroidetes phylum, a group of gram-negative bacteria that make up a major part of the mammalian distal intestinal microbiota [98]. To validate the results obtained by sequencing, we determined the relative concentrations of Firmicutes and Bacteroidetes with real-time PCR. The Firmicutes/Bacteroidetes ratio for faecal samples of B1 and B2 was 1/0.0004 and 1/0.0081, respectively, indicating a very low abundance of Bacteroidetes. In spiked faecal samples, however, *Bacteroides* spp. were succesfully recovered down to 1% (10^4CFU/mL).

Taxonomic assignment at family level revealed 16 different families of which *Clostridiaceae*, *Ruminococcaceae*, *Peptococcaceae* and the unclassified Clostridiales Incertae Sedis XIV held most representatives. Of all these families, the *Clostridiaceae* represented by far the highest number of different phylotypes (**FIG 4.1**). The distribution of common OTUs within the predominant bacterial families confirms the phylotype richness of *Clostridiaceae* in both libraries (**TABLE 4.1**).

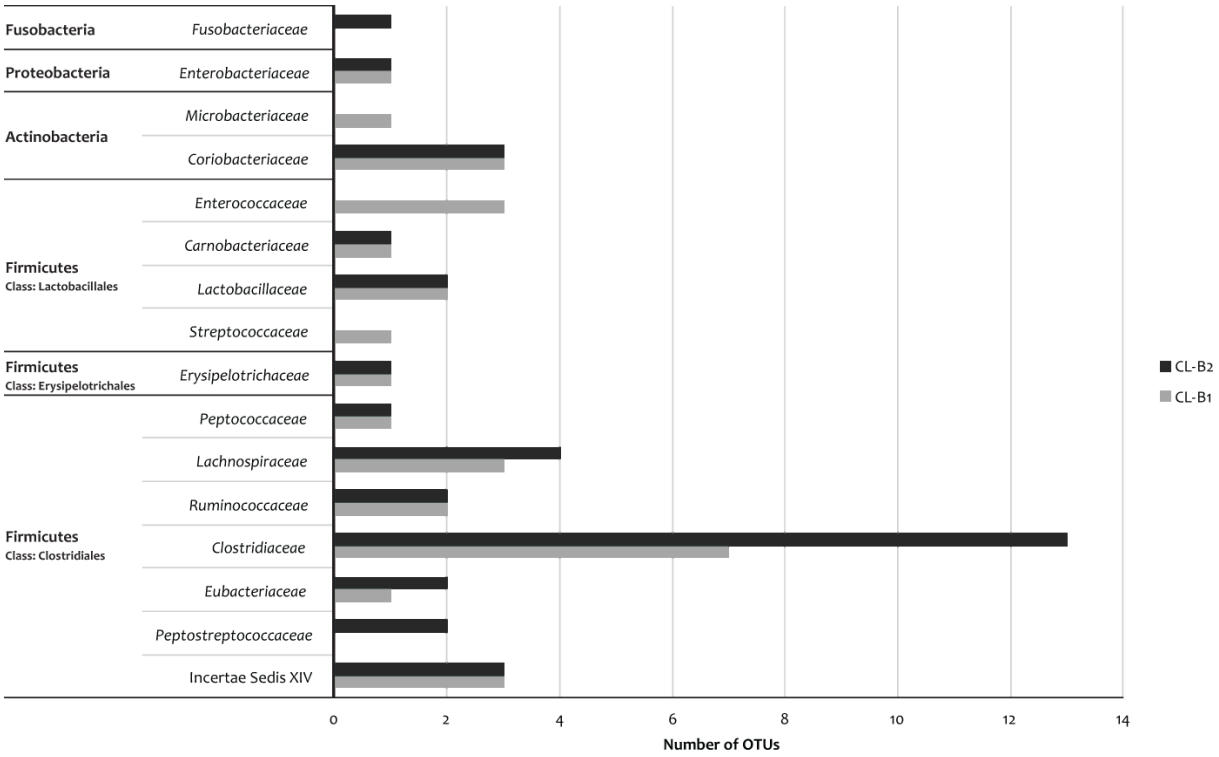

FIG 4.1 | Phylotype frequency at the family level as revealed by clone library analysis of captive cheetah faeces. Samples from two 10-year old male cheetahs which were housed together and fed the same diet.

4.3.3. Phylogenetic analysis of 16S rRNA gene clone libraries at OTU level

For each OTU, a representative clone sequence was selected along with the type strain of its nearest validated species neighbour as obtained in RDP to construct a wide-range phylogenetic tree. **FIG 4.2** shows the phylogenetic inferences among the OTUs affiliated with the phyla Firmicutes, Actinobacteria, Proteobacteria and Fusobacteria. Recovered sequences within the Firmicutes spanned three major orders *i.e.* Clostridiales, Lactobacillales and Erysipelotrichales.

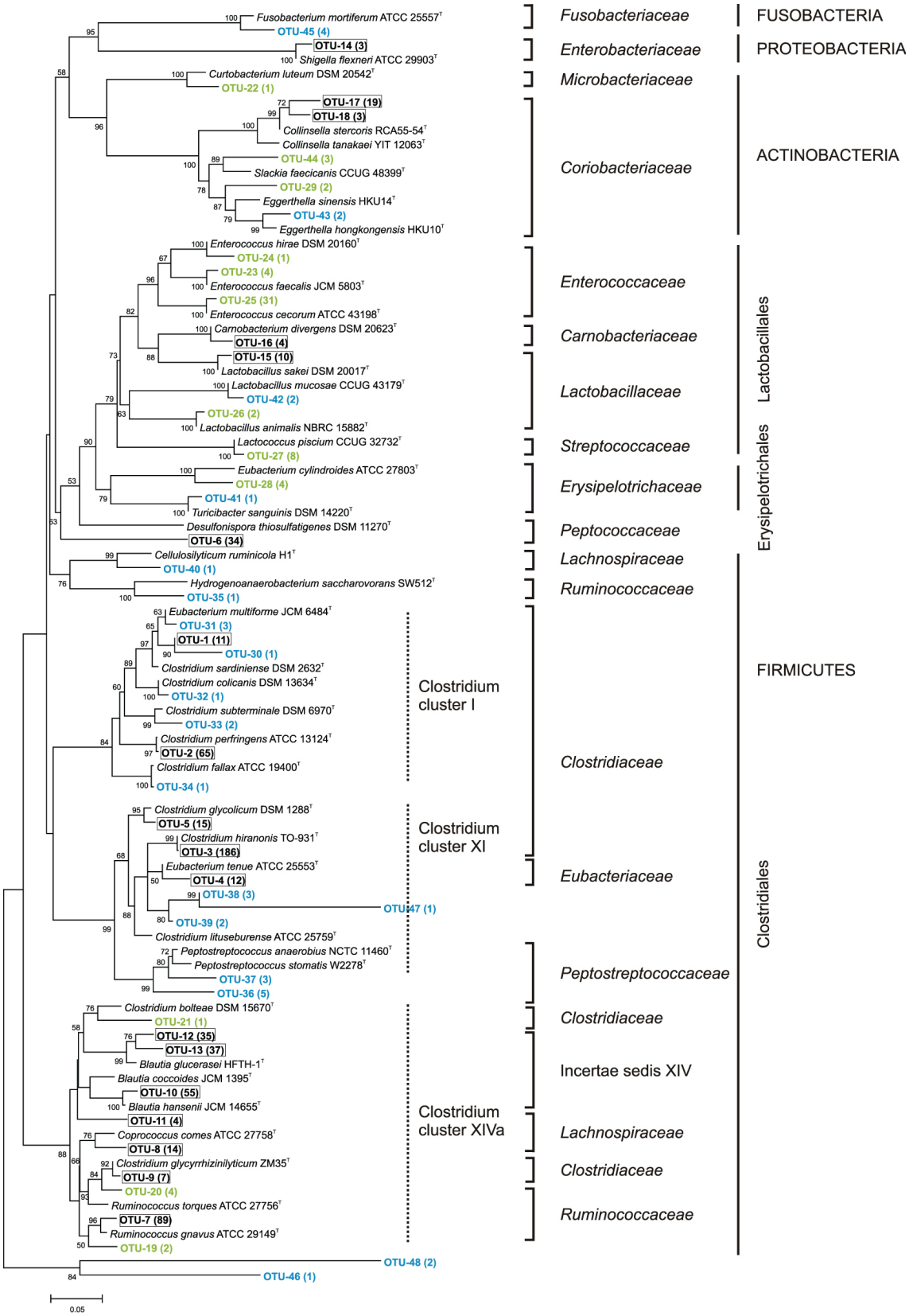

FIG 4.2 | Neighbour-joining phylogenetic tree showing the nearest phylogenetic related type strains for recovered OTUs from two 16S rRNA gene clone libraries from captive cheetah faeces. Bootstrap values, expressed as percentages of 1000 replications, above 50% are given at branching points. The scale 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 (blue)

Most of the clones fell within the Clostridiales, representing members of seven different bacterial families. A total of 186 clones of this class (31%) belonged to OTU-3 and were highly related (<1% nucleotide divergence) to *Clostridium hiranonis* TO-931^T. Within the *Clostridiaceae* a high nucleotide similarity was also found for OTU-2, which grouped 65 clones closely to *Clostridium perfringens* ATCC 13124^T, and for OTU-34, which clustered with *Clostridium fallax* ATCC 19400^T. However, the latter only consisted of one clone and displayed a low bootstrap value of 56% at its node. For OTU-9, OTU-32 and OTU-5, high bootstrap values (92%, 100% and 95%) and a low nucleotide divergence (1%) indicated their close phylogenetic affiliation to *Clostridium glycyrrhizinilyticum* ZM35^T, *Clostridium colicanis* 13634^T and *Clostridium glycolicum* DSM 1288^T, respectively. The remaining five OTUs within the *Clostridiaceae* family (OTU-31, OTU-1, OTU-30, OTU-33 and OTU-21) clustered under lower bootstrap values with their respective type strains. The *Ruminococcaceae* family was also well represented by four OTUs of which OTU-7 constituted 89 clones closely related to *Ruminococcus gnavus* ATCC 29149^T. The high bootstrap value (100%) at the node of cluster OTU-35 and *Hydrogenoanaerobacterium saccharovorans* SW512^T suggests a reliable phylogenetic positioning although there was less than 90% sequence similarity between both. The remaining OTU-19 and OTU-20 included only 6 clones clustering at 5% nucleotide divergence with *Ruminococcus gnavus* ATCC 29149^T and *Ruminococcus torques* ATCC 27756^T, respectively. The *Peptococcaceae* family was only represented by OTU-6, which included 34 clones and exhibited a low sequence similarity (80%) with the nearest type strain, *Desulfonispora thiosulfatigenes* DSM 11270^T. Moreover, the low bootstrap value (63%) questions the phylogenetic position of OTU-6 in this tree. The remaining families *Lachnospiraceae*, *Enterococcaceae* and *Peptostreptococcaceae* were represented by 6 different OTUs which together encompassed 6% of all sequences allocated to the Clostridiales. The unclassified Clostridiales, Incertae Sedis XIV, harbored 18% of all sequences across three OTUs and were all affiliated to the genus *Blautia*. However, only OTU-10 showed 1% sequence divergence to its type strain *Blautia hansenii* JCM 14655^T, whereas OTU-12 and OTU-13 differed at least 4% from the closest relative *Blautia glucerasei* HFTH-1^T. Based upon the previously proposed classification of *Clostridium* spp. in phylogenetic clusters [327], Clostridiales sequences from this study fell into three clusters. These included *Clostridium* cluster XIVa (43%), which showed the highest OTU variety containing OTU-7 to OTU-13, *Clostridium* cluster XI (38%) and *Clostridium* cluster I (13%).

Within the Lactobacillales, the bootstrap value of 79% at the node tenuously supports the grouping in four families. Three OTUs together represented by 36 clones grouped in the *Enterococcaceae*. Of these, OTU-24 was closely related to *Enterococcus hirae* DSM 20160^T although it only represented one clone with a 3% nucleotide divergence. The other two OTUs (OTU-23 and OTU-25) differed only 1% from the sequences of *Enterococcus faecalis* JCM 5803^T and *Enterococcus cecorum* ATCC 43198^T, respectively. For the *Carnobacteriaceae*, a monophyletic branch at 100% bootstrap support was formed by OTU-16 with *Carnobacterium divergens* DSM 20623^T. A total of 14 clones all grouping in the *Lactobacillaceae* formed three subclusters, each at 100% bootstrap support with their closest type strain. OTU-15 was phylogenetically linked to *Lactobacillus sakei* DSM 20017^T, OTU-42 to *Lactobacillus mucosae* CCUG 43179^T

and OTU-26 to *Lactobacillus animalis* NBRC 15882^T. Finally, *Streptococcaceae* were represented by OTU-27, which was closely related (1% nucleotide divergence) to *Lactococcus piscium* CCUG 32732^T.

The order Erysipelotrichales was divided into two distinct clusters representing members of the *Erysipelotrichaceae* family. More specifically, OTU-28 (4 clones) grouped most closely to *Eubacterium cylindroides* ATCC 27803^T, whereas the single clone of OTU-41 clustered with *Turicibacter sanguinis* MOL 361^T.

The branching pattern within the phylum Actinobacteria consisted of two families. The *Microbacteriaceae* were represented by a single clone (OTU-22) clustering at 100% bootstrap support with *Curtobacterium luteum* DSM 20542^T. The *Coriobacteriaceae* comprising the genera *Collinsella*, *Slackia* and *Eggerthella* were represented by five OTUs. Of these, OTU-17 (19 clones) and OTU-18 (3 clones) clustered with *Collinsella stercoris* RCA55-54^T and *Collinsella tanakaei* YIT 12063^T, respectively. The few clones assigned to OTU-29, OTU-43 and OTU-44 were most closely related to *Eggerthella hongkongenis* HKU10^T, *Eggerthella sinensis* HKU14^T and *Slackia faecicanis* CCUG 48399^T, respectively.

The single OTU belonging to the Proteobacteria, OTU-14 (3 clones), exhibited <2% nucleotide divergence with *Shigella flexneri* ATCC 29903^T with 100% bootstrap support. Likewise, the phylum Fusobacteria was only represented by OTU-45 (4 clones), which was phylogenetically most closely related to *Fusobacterium mortiferum* ATCC 25557^T.

Five OTUs (OTU-38, OTU-39, OTU-46, OTU-47, OTU-48), containing 1 to 3 clones each, failed to clearly group within a particular genus or family. Given that all sequences used for phylogenetic analyses were of good quality, these OTUs may represent species that are currently not included in the RDP database.

4.3.4. Common diversity of CL-B1 and CL-B2

The faecal community members shared by CL-B1 and CL-B2 encompassed three phyla (Firmicutes, Actinobacteria and Proteobacteria), 10 families and 18 OTUs (OTU-1 to OTU-18). The *Clostridiaceae* family harbored five common OTUs. Of these, OTU-3 (affiliated with *Clostridium hiranonis* TO-931^T) accounted for 13.6% and 39.4% of all clones in CL-B1 and CL-B2, respectively. Followed by OTU-7 (affiliated with *Ruminococcus gnavus* ATCC 29149^T) representing 19.6% and 5.7% of all sequences in CL-B1 and CL-B2, respectively (**TABLE 4.1**). On top of the five common OTUs, CL-B2 harbored eight unique OTUs within the family *Clostridiaceae* compared to one unique OTU (OTU-21) for CL-B1. Other shared families within the phylum Firmicutes were the *Peptococcaceae*, *Eubacteriaceae*, *Lachnospiraceae* and unclassified Clostridiales. All of these consisted of common OTUs with the exception of the *Lachnospiraceae* family that also comprised a single clone of OTU-40 in CL-B2. However, the phylogenetic position of OTU-40 displayed 8% nucleotide divergence with the closest type strain, *Cellulosilyticum ruminicola* H1^T. In the Proteobacteria, only the family *Enterobacteriaceae* was represented with a single common OTU-14 (affiliated with *Shigella flexneri* ATCC 29903^T), which harbored a minority population of three clones. The phylum Actinobacteria was represented by two common OTUs (OTU-17 and OTU-18) that were phylogenetically related to the *Coriobacteriaceae*.

TABLE 4.1 | Most abundant OTUs, their taxonomic assignment at family level and closest type strain in number and % of clones for both clone libraries from captive cheetah faeces.

OTU[a]	Bacterial family	*Clostridium* cluster	Closest type strain	CL-B1 (352 clones)	CL-B2 (350 clones)
OTU-2	***Clostridiaceae***	I	*Clostridium perfringens* ATCC 13124^T	6 (1.7%)	59 (16.9%)
OTU-3	***Clostridiaceae***	XI	*Clostridium hiranonis* TO-931^T	48 (13.6%)	138 (39.4%)
OTU-5	***Clostridiaceae***	XI	*Clostridium glycolicum* DSM 1288^T	1 (0.3%)	14 (4.0%)
OTU-6	***Peptococcaceae***	n/a	*Desulfonispora thiosulfatigenes* DSM 11270^T	33 (9.4%)	1 (0.3%)
OTU-7	***Ruminococcaceae***	XIVa	*Ruminococcus gnavus* ATCC 29149^T	69 (19.6%)	20 (5.7%)
OTU-10	**Incertae Sedis XIV**	XIVa	*Blautia hansenii* JCM 14655^T	36 (10.2%)	19 (5.4%)
OTU-12	**Incertae Sedis XIV**	XIVa	*Blautia glucerasei* HFTH-1^T	32 (9.1%)	3 (0.9%)
OTU-13	**Incertae Sedis XIV**	XIVa	*Blautia glucerasei* HFTH-1^T	29 (8.2%)	8 (2.3%)
OTU-17	***Coriobacteriaceae***	n/a	*Collinsella stercoris* RCA55-54^T	6 (1.7%)	13 (3.7%)
OTU-25	***Enterococcaceae***	n/a	*Enterococcus cecorum* ATCC 43198^T	31 (8.8%)	-

[a] OTUs which consist of at least ≥ 10 clones in CL-B1 or CL-B2; OTU = operational taxonomic unit; n/a = not applicable

4.3.5. Comparison with available 16S rRNA sequences from captive cheetahs

Our dataset of 702 quality-checked sequences was compared with 597 full-length 16S rRNA gene sequences retrieved from a large comparative microbiome study of Ley and co-workers [86] in which one faecal sample each of two captive cheetahs from Saint Louis Zoo (St Louis, Missouri, USA) were included. Despite differences in sequence number and sequence length, both datasets were compared with taxonomic RDP annotation. In line with the present study, Bacteroidetes represented only a very marginal share (*i.e.* 1.3%) in Ley *et al.*'s dataset. At family level, the dominance of *Clostridiaceae* (16.5%) and *Ruminococcaceae* (4.0%) members was also confirmed. The share of *Peptococcaceae* (1.7%) and the unclassified Clostridiales Incertae Sedis (0.8%) in Ley *et al.*'s dataset was considerably lower compared to our dataset (5% and 18%, respectively). Two other bacterial families, also represented in the dataset of this study, made up a big part of Ley *et al.*'s dataset, *Peptostreptococcaceae* (13%) and *Lachnospiraceae* (11%). Taken together, only the *Clostridiaceae*, *Lactobacillacea*e and *Erysipelotrichaceae* families were common to the faecal microbiota of all four cheetahs included in these two studies.

4.4. Discussion

This study set out to determine the predominant faecal microbial communities of captive cheetahs using 16S rRNA gene clone libraries. At the onset of the study, only two animals with well-documented dietary and health records and housed according to EAZA standards were available for this study in Flanders, Belgium. Phylogenetic analysis of the pooled library set revealed a highly complex microbiota covering a broad phylogenetic spectrum. The Firmicutes were by far the most abundant bacterial phylum compared to the minority of Actinobacteria, Fusobacteria and Proteobacteria. Surprisingly, none of the OTUs of both clone libraries were assigned to members of the Bacteroidetes, the phylum that together with the Firmicutes accounts for >98% of the 16S rRNA gene sequences detected in the gut microbiota of vertebrates [311]. The Bacteroidetes comprise important degraders of complex and otherwise indigestible dietary polysaccharides in the large intestine, which leads to the production of short-chain fatty acids that are reabsorbed by the host as an energy source [328,329]. Using a variety of methods, Bacteroidetes have been identified as a dominant group in the faecal microbiota of dogs (27-34%) fed experimental diets (30% protein and 20% fat) [258,330], wild wolves (16.9%) feeding on raw meat [259] and grizzly bears (40%) on an omnivorous diet [268]. Feline microbiome studies using 16S rRNA clone libraries or pyrosequencing have also reported that Bacteroidetes is one of the major (0.45%-10%) phyla in the faecal microbiota of cats alongside Firmicutes and Actinobacteria [285,331]. A recent study using 454 pyrosequencing even reported Bacteroidetes to be the most predominant (68%) bacterial phylum in the intestinal microbiome of domestic cats [287]. Although relative levels of the dominant phyla in cats seem to vary between studies, likely as a result of differences in methodologies and/or in dietary regimes of the studied cats, one could expect to also find Bacteroidetes in most other felids. The complete absence of Bacteroidetes members in the 16S rRNA clone libraries of the two captive cheetahs contradicts this expectation, but was corroborated by real-time PCR data indicating a hardly detectable concentration of this phylum against a high background of Firmicutes. The finding that *Bacteroides* spp. could be detected in spiked faecal samples at 10^4 CFU/ml and possibly lower, excludes major detection artefacts introduced during DNA extraction. Further support for our observations are provided by a comparative study of the gut-associated bacterial communities in 60 mammalian species showing that Bacteroidetes is a rare phylum in most carnivores [86]. In that study, 1-3% of the 16S rRNA gene sequences of captive lions, and up to 15% of the 16S rRNA gene sequences of hyaenas and bushdogs were phylogenetically linked to Bacteroidetes, whereas only a marginal contribution (<1%) of this phylum was found for captive polar bears and cheetahs. This is comparable to Bacteroidetes levels reported in a recent microbiome study of captive polar bears [332] and our findings for captive cheetahs. The common denominator between the latter two strict carnivores is their protein-rich diet, whereas domestic cats are usually fed commercially prepared diets containing moderate quantities of carbohydrates and plant-derived soluble fibres [249]. This seems to suggest that differences in dietary regimes and feeding habits account for the large variation in Bacteroidetes levels among carnivores. Low proportions of Bacteroidetes have also been reported in giant pandas which

belong to the order Carnivora and have a simple intestinal tract, but are feeding on bamboo [269]. Despite their herbivorous lifestyle, studies have shown that the panda faecal microbiota is more similar to other Carnivora than to unrelated herbivores suggesting that next to diet also gut physiology is a regulator of the faecal microbiota composition [86,311].

Within the Firmicutes, the majority of the Clostridiales isolates common to both clone libraries was assigned to *Clostridium* clusters XIVa (43%), XI (38%) and I (13%). Our results are consistent with previous studies that reported a high prevalence of these three *Clostridium* clusters in carnivores [260,265]. Likewise, similar distributions were found in feline microbiome studies using 16S rRNA clone libraries [282,331] or 16S rRNA gene pyrosequencing [285]. Also in the two cheetahs studied by Ley and co-workers [86], similar high abundances of *Clostridium* clusters XIVa and XI were found in two other cheetahs. *Clostridium* cluster XIVa constitutes a major and highly diverse bacterial group in the distal intestines of mammals [333]. This phylogenetically heterogeneous cluster is in both clone libraries represented by *Ruminococcaceae* spp. most closely related to known mucin-degrading organisms such as *Ruminococcus torques* and *Ruminococcus gnavus* [334] as well as members of the recently proposed genus *Blautia* [335]. The latter group comprises important producers of short-chain fatty acids such as butyrate, which is an important source of energy for colonic epithelial cells and has been shown to possess anti-inflammatory and anticarcinogenic potential [336,337]. Feline and canine inflammatory bowel diseases have been associated with reduced bacterial species richness and a reduced proportion of *Clostridium* cluster XIVa [281,294,295]. It is noteworthy that the two cheetahs included in our study showed no signs of gastrointestinal disease. *Clostridium* clusters XI and I include saccharolytic fibre-fermenting species but also proteolytic or toxinogenic clostridia [327]. In *Clostridium* cluster XI, 87% of the common sequences displayed >99% sequence similarity to the type strain of *Clostridium hiranonis*. This species was first described in human faeces and displays bile acid 7-α-dehydroxylating activity. In addition, acetic acid and minor amounts of propionic acid and iso-butyric acid are produced from mono- and disaccharides [338]. Ritchie and co-workers [331] found *Clostridium* cluster XI to account for 22% of the faecal microbiota in healthy cats. Up to 86% of the clones assigned to *Clostridium* cluster I in our study were phylogenetically most closely related to the type strain of the potentially pathogenic species *Clostridium perfringens*. However, with reported isolation rates of up to 63% in healthy cats [290], *C. perfringens* should probably be considered as a common commensal of the feline intestine. Moreover, no significant differences in prevalence of either *C. perfringens* or toxigenic *C. perfringens* strains were observed between healthy cats and cats with diarrhoea [290].

Protein-rich diets may increase the presence of *Clostridium* cluster I in pet cats and dogs and induce a shift towards a higher prevalence of proteolytic bacterial species [314,339]. A similar dietary influence has also been reported in other carnivores. *Clostridium* cluster I and XI prevailed in polar bears feeding on seals and fish [332] and captive grizzly bears feeding on a regular diet containing up to 31% protein, 15-18% fat and 0.37% fibre [265]. The latter study indicated that captive grizzly bears consuming a protein-based diet were more prone to carry *C. perfringens* than wild grizzly bears consuming a more plant-

based diet. These results suggest a positive correlation between the prevalence of *Clostridium* clusters I and XI and dietary protein content. In the present study, both cheetahs included in our study were fed a protein-rich diet with minimal dietary fibre *i.e.* boneless horsemeat. Therefore, the high proportions of *Clostridium* cluster I and XI in the faecal microbiota of captive cheetahs may be a reflection of their dietary habits.

Common bacterial communities classified in the phylum Actinobacteria harbored solely species belonging to the genus *Collinsella* within the *Coriobacteriaceae*. This family is a frequent resident of the feline gut microbiota [272]. No members were identified of the *Bifidobacteriaceae*, a group of fibre-fermenting gut bacteria that largely contribute to cross-feeding mechanisms leading to the production of butyrate [138,340]. Also in two other studies both using 16S rRNA gene clone libraries to study the faecal microbiota of wild wolves [259] and pet cats [282], no Bifidobacteriaceae were encountered. In contrast, other studies have reported the presence of *Bifidobacteriaceae* in the feline faecal microbiota using alternative techniques such as culturing [278], FISH [281] and a chaperonin 60 gene-based clone library [283]. This suggests that differences in methodologies may, at least to some extent, explain the observed differences between studies. In fact, it has been shown that *Bifidobacteriaceae* may be underrepresented in 16S rRNA gene-based studies, possibly due to the use of universal primers that may underestimate the GC-rich Actinobacteria. Therefore, the combined use of universal and genus-specific primers has been suggested to characterize *Bifidobacterium* spp. in intestinal microbiota [331,341,342]. In the present study, real-time PCR enumeration of *Bifidobacterium* revealed a low mean $\log_{10}$ number of 4.43. On the one hand, this illustrates the inability of the clone library approach to detect low levels of *Bifidobacterium* in the cheetah faecal samples. On the other hand, the finding of a significantly higher mean $\log_{10}$ *Bifidobacterium* concentration of 9.13 in faecal samples of five domestic cats with the same real-time PCR protocol indicates that marked differences exist in bifidobacterial levels of cheetahs and domestic cats. Possibly, these differences reflect the strictly carnivorous diet of captive cheetahs. In fact, *Bifidobacteriaceae* have been negatively correlated with the protein content of the diet [314,343] and only a few studies have reported the presence of bifidobacteria in faeces of carnivores [344].

Finally, the minor share of Fusobacteria and Proteobacteria found in this study is also confirmed in other feline microbiome studies using 16S rRNA gene clone libraries [282] or shotgun sequencing [287]. Felids seem to harbor less Proteobacteria and Fusobacteria compared to other carnivores such as wolves [259] and dogs. In the latter species, even substantial numbers of Fusobacteria have been observed, but the significance of an enriched Fusobacteria population is yet unknown [330]. In the Proteobacteria, a minority of three clones affiliated with *Shigella flexneri* ATCC 29903^T. This species is principally a primate pathogen causing bacillary dysentery or shigellosis [345]. Cats have not been reported to be naturally infected [346], although these organisms may be transiently excreted in some clinically normal domestic cats [287,331]. The two cheetahs included in this study showed no signs of shigellosis and to our knowledge this type of infection has not been reported in cheetahs thus far.

4.5. Conclusions

This is the first ever study to specifically characterize the predominant faecal bacterial populations of captive cheetahs using a combination of 16S rRNA clone library and real-time PCR analyses. The study revealed a complex microbial diversity predominantly composed of Firmicutes. The abundance of *Clostridium* clusters XIVa, XI and I in this phylum resembles that in the faecal microbiota of other carnivores. However, the near absence of Bacteroidetes and the low abundance of *Bifidobacteriaceae* are in sharp contrast with the situation in domestic cats but in agreement with faecal microbiota composition reported in other Carnivora. Thus, in addition to the apparent differences in feeding habits between both felid species, also our microbiological findings thus question the role of the domestic cat as a suitable model for nutritional intervention studies in captive felids such as cheetahs.

The present study provides a first taxonomic baseline for further characterizations of the diversity and dynamics of the cheetah intestinal ecosystem. To confirm our main findings based on two animals, the collection of fresh and well-documented faecal samples from more captive cheetahs worldwide is the next challenge. Ultimately, the resulting microbial insights may contribute to the optimization of feeding strategies and the improvement of the general health status of cheetahs in captivity.

SUPPLEMENTARY DATA

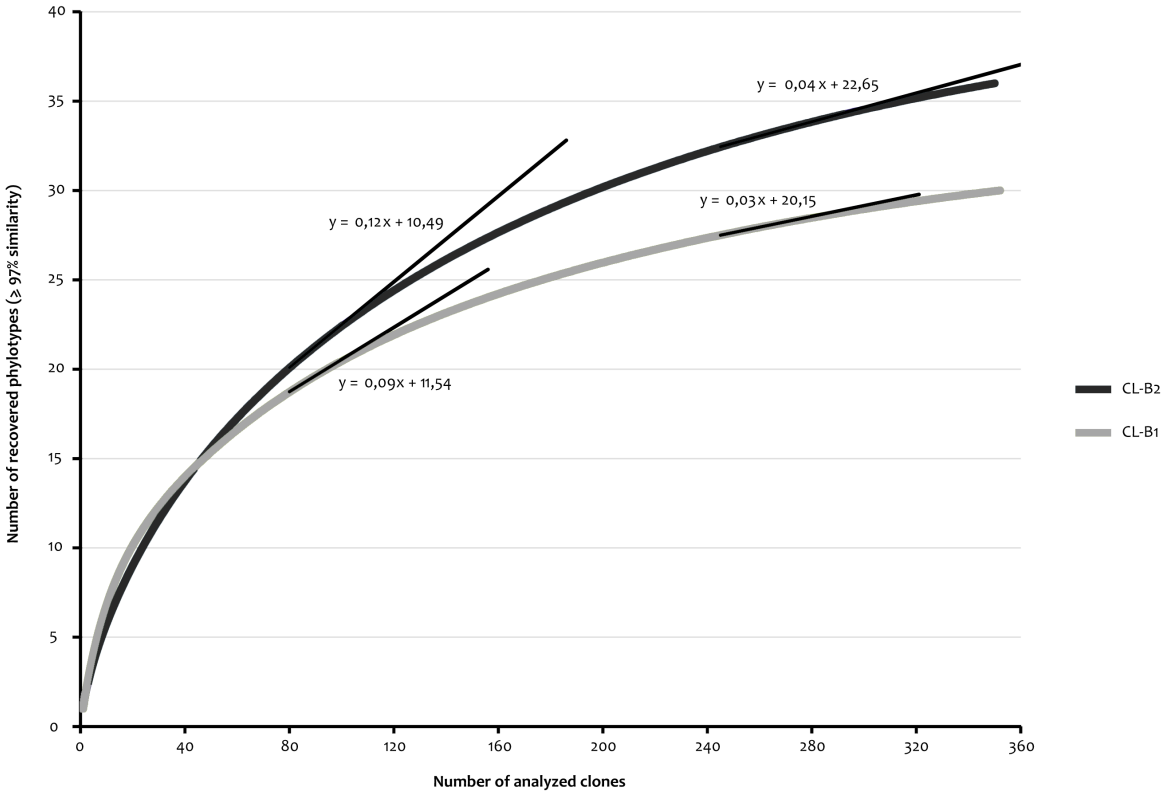

FIG S4.1 | Rarefaction curves for bacterial 16S rRNA gene sequences obtained by clone library analysis of captive cheetah faecal samples. The slopes of corresponding lineair lines indicate a flattening of the rarefaction curves. CL-B1: clone library of faecal samples of captive cheetah B1; CL-B2: clone library of faecal samples of captive cheetah B2

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5 INTEGRATED COMMUNITY PROFILING INDICATES LONG-TERM TEMPORAL STABILITY OF THE PREDOMINANT FAECAL MICROBIOTA IN CAPTIVE CHEETAHS

Understanding the symbiotic relationship between gut microbes and their animal host requires characterization of the core microbiota across populations and in time. Especially in captive populations of endangered wildlife species such as the cheetah (*Acinonyx jubatus*), this knowledge is a key element needed to enhance feeding strategies and reduce gastrointestinal disorders. In order to investigate the temporal stability of the intestinal microbiota in cheetahs under human care, we conducted a longitudinal study over a 3-year period with bimonthly faecal sampling of five cheetahs housed in two European zoos. For this purpose, an integrated 16S rRNA DGGE-clone library approach was used in combination with a series of real-time PCR assays. Our findings disclosed a stable faecal microbiota, beyond intestinal community variations that were detected between zoo sample sets or between animals. The core of this microbiota was dominated by members of *Clostridium* clusters I, XI and XIVa, with mean concentrations ranging from 7.5-9.2 log10 CFU/g faeces and with significant positive correlations between these clusters ($P<0.05$), and by *Lactobacillaceae*. Moving window analysis of DGGE profiles revealed 23.3-25.6% change between consecutive samples for four of the cheetahs. The fifth animal in the study suffered from intermediate episodes of vomiting and diarrhoea during the monitoring period and exhibited remarkably more change (39.4%). This observation may reflect the temporary impact of perturbations such as the animal's compromised health, antibiotic administration or a combination thereof, which temporarily altered the relative proportions of *Clostridium* clusters I and XIVa. In conclusion, this first long-term monitoring study of the faecal microbiota in feline strict carnivores not only reveals a remarkable compositional stability of this ecosystem, but also shows a qualitative and quantitative similarity in a defined set of faecal bacterial lineages across the five animals under study that may typify the core phylogenetic microbiome of cheetahs.

5.1. Background

As first shown in human studies and later also confirmed for other animal species, diet is considered a major regulator of the intestinal microbial composition and metabolic homeostasis in all mammals [86]. Also in zoo animal husbandry, nutrition is one of the most critical components [302], to the extent that feeding mismanagement is recognized as a major risk factor for suboptimal health, low breeding performance and a higher incidence of gastrointestinal and metabolic diseases [303,347]. The latter is especially true for endangered strict carnivores such as the cheetah (*Acinonyx jubatus*) where high vulnerability to gastrointestinal disorders in captivity is a major concern [32,34].

Conservation programs would clearly benefit from a finer scale understanding of the relationship between food availability (e.g. fasting frequency), gut physiology and symbiotic gut microbes in cheetahs [348–350]. Also, such information may prove to be a key component to enhancing feeding strategies for disease prevention or therapeutic intervention [98,265,286]. Due to its close phylogenetic relationship and the fact that it is a far better studied and accessible species, the domestic cat has been proposed as reference model for dietary intervention studies in exotic wild felids kept under human care such as cheetahs [306]. However, the scientific rationale of this proposal is questionable because dietary regimes and feeding habits of the domestic cat and wild feline species are markedly different, and this is likely to affect the taxonomic structure of their gastrointestinal (GI) microbiota as well. In fact, a recent study characterizing the faecal microbiota of two cheetahs under care in a Belgian zoo revealed a pronounced underrepresentation of *Bifidobacteriaceae* and Bacteroidetes members compared to domestic cats [351]. These data thus highlight the need for a more advanced and fundamental understanding of the cheetah's GI microbiota itself instead of focussing on extrapolation from genetically related species that evolved away from a strict carnivorous diet as a result of domestication. However, before specific dietary applications can be developed in any population of captive cheetahs in the long term, it is of particular relevance to assess the cross-sectional and longitudinal variation within their intestinal ecosystem and to identify its potential core members [89,352].

Recently, we provided a first taxonomic baseline of the predominant faecal bacterial populations based on single samples of two captive cheetahs from the same zoo [351]. The aim of this follow-up study was to address the temporal stability of the intestinal microbiota of captive cheetahs by including more animals from different zoos sampled at multiple time points. To this end, we carried out a longitudinal study with bimonthly faecal sampling of 5 cheetahs housed in two European zoos over a 3-year period. An integrated 16S rRNA DGGE-clone library approach was used in combination with real-time PCR assays to monitor microbial community variation and diversity in the time series samples.

5.2. Material & Methods

Ethics statement

This study was conducted non-invasively, without animal handling or any change in daily management and housing conditions of the animals. Faecal samples were collected from two adult cheetahs housed at Zoo Parc Planckendael (Belgium) and three adult cheetahs housed at Zoo Parc Overloon (The Netherlands) under supervision of the zookeepers. Both zoos are recognized full members of the European Association of Zoos and Aquaria (EAZA, http://www.eaza.net/membership) and the cheetahs are housed according to the Minimum Standards for the Accommodation and Care of Animals in Zoos and Aquaria and the EAZA Code of Practice. Permission for faecal sampling was obtained from the staff veterinarians and mammal curators. Ghent University Animal Ethics Committee approval and any additional permits were not further needed.

Animals & diet

At Zoo Parc Planckendael (PL; 50° 99' N, 4° 52' E) in Belgium, the two male cheetahs (B1 and B2; born in 2001) shared indoor and outdoor housing and were fed their regular zoo diet consisting of chunked boneless horsemeat (2 kg/day/animal) topdressed with a vitamin and mineral premix (Carnicon®; Aveve, Leuven, Belgium) and once or twice a month interspersed with unsupplemented whole rabbits. No medical or health problems were reported or apparent on remote examination, and both cheetahs were treated prophylactically for internal parasites (Horseminth®; Pfizer, Brussels, Belgium) approximately every two months during the entire study period starting one week before first sampling (PL-T1).

At Zoo Parc Overloon (OV; 51° 57' N, 5° 94' E) in the Netherlands, the two male cheetahs (NL9 and NL10; respectively born in 2000 and 2002) shared indoor and outdoor housing whereas the female cheetah (NL11; born in 2005) was housed separately. During the study, animal NL11 gave birth to four cubs. From sampling point OV-T4 onwards, NL11 shared indoor and outdoor housing with her litter of which two survived the following year. All cheetahs from OV were fed their regular zoo diet consisting of unsupplemented whole rabbits randomly interspersed with chicken, vitamin and mineral supplemented chunked boneless horsemeat (1.5 kg/day/animal) or pieces of beef, horse and antelope carcasses. The three cheetahs were treated prophylactically roughly on a three monthly basis for internal parasites (Drontal® Cat; Bayer, Mijdrecht, the Netherlands), *i.e.* one month before first sampling OV-T1, between OV-T2 & OV-T3, OV-T5 & OV-T6, OV-T10 & OV-T11, OV-T11 & OV-T12 and OV-T12 & OV-T13. Interim vomiting, diarrhoea, loss of appetite and overall weakness were reported for animal NL9 between the time of sampling points OV-T2 and OV-T5. Both male cheetahs were also relocated and left the study at sampling point OV-T11. The typical diet of each animal was consistent throughout the 3-year study period.

Sample collection

Over a 3-year period starting in October 2010 in PL and May 2011 in OV, cheetahs were bimonthly monitored for 12h to collect animal-specific faecal samples immediately upon defaecation. This way, a total of 55 fresh faecal samples (100-200 gram/animal) were collected. At PL, monitoring during 16 different time points (PL-T1 to PL-T16) resulted in 14 samples each from animals B1 and B2. At OV, 8 faecal samples were collected from animal NL9, 9 from animal NL10 and 10 from animal NL11 at 14 different time points (OV-T1 to OV-T14). For a number of time points samples are missing because no defaecation was observed. All samples were scored for consistency [353], aliquoted into plastic tubes, transported on dry ice and stored at -80°C until further analysis. In addition, each animal's medical history and the dietary regime *i.e.* the diet type (type of meat or prey) and fasting days were recorded on a daily basis.

DNA extraction

Prior to DNA extraction, 25 grams (wet weight) of each thawed faecal sample was placed separately in sterile stomacher bags and homogenised in 225 ml peptone-buffered saline (PBS) (0.1% [wt/vol] bacteriological peptone [L37; Oxoid, Basingstoke, United Kingdom], 0.85% [wt/vol] NaCl [106404; Merck, Darmstadt, Germany]). The sludgy homogenate was filtered on a Büchner funnel to discard large particles such as hair and bones, and subsequently divided into 1.5 ml aliquots which were stored at -80°C.

The protocol of Pitcher *et al.* (1989) [315] was used in a modified version [210] to extract total bacterial DNA from the faecal samples. DNA size and integrity were assessed on 1% agarose electrophoresis gels stained with ethidium bromide. DNA concentration and purity were determined by spectrophotometric measurements at 234, 260 and 280 nm. DNA extracts were finally diluted to OD 1 with TE buffer (1 mM EDTA [324503; Merck, Darmstadt, Germany], 10 mM Tris-HCl [648317; Merck, Darmstadt, Germany]) and stored at -20°C.

Community PCR for Denaturing Gradient Gel Electrophoresis (DGGE)

The variable V3 region of the 16S rRNA gene was amplified using the universal bacterial primers GC-F357 (5'-CCTACGGGAGGCAGCAG-3') and R518 (5'-ATTACCGCGGCTGCTGG-3') [179,354]. PCR was performed with a *Taq* polymerase kit (Applied Biosystems, Gent, Belgium). The PCR reaction mix included: 6 µl 10x PCR buffer (containing 15 mM $MgCl_2$), 2.5 µl bovine serum albumin (0.1 mg/ml), 2.5 µl 2 mM dNTPs, 2 µl of each primer (5 µM), 0.25 µl *Taq* polymerase, 33.75 µl milliQ water and 1 µl 10-fold diluted DNA solution. All reactions were carried out in a final volume of 50 µl using the following PCR programme: initial denaturation at 95°C for 1 min, 30 cycles of 95°C for 30 s, 55°C for 45 s and 72°C for 1 min, and final extension of 72°C for 7 min. Negative (milliQ water as template) and positive controls were included in parallel. Amplicons were checked on a 1% agarose gel under UV illumination after ethidium bromide staining. PCR amplification products were stored at -20°C until DGGE analysis.

DGGE analysis and gel processing

The resulting 16S rRNA amplicons were analyzed with DGGE fingerprinting (D-code System, Bio-Rad, Nazareth, Belgium) using a 35-70% denaturing gradient as previously described [354]. Per lane, 25 µl of PCR product was loaded and electrophoresis was performed in 1x Tris-Acetate-EDTA buffer (TAE, catalog no. 161-077, Bio-Rad) for 990 min at a constant voltage of 770 V. Afterwards, DGGE gels were stained for 30 min with 1x SYBR®Gold nucleic acid gel stain (S-11494, Invitrogen, Merelbeke, Belgium) and band profiles were visualized using a charge-coupled device (CCD) camera and the Bio-Rad Quantity One software program.

A standard reference lane containing the V3-16S rRNA amplicons of 12 taxonomically well-characterized bacterial species [354] was included every fifth lane for normalization of the fingerprint profiles using the BioNumerics software version 7.0 (Applied Maths, St-Martens Latem, Belgium). After normalization of the gels, individual bands were marked using the auto search bands option, followed by manual correction if necessary. Band intensities were calculated from the peak-area in the densitometric curves. All of the profiles were compared using the band-matching tool, and uncertain bands were included in the position tolerance settings. Bands were allocated to band-classes (Bcl) which are arbitrarily generated in a collective analysis of all 55 profiles by tracing common bands across different sample profiles. A maximum deviation of 0.5% was applied, which means that allocation of a band to a specific Bcl was only allowed if it was located at a distance less than 0.5% of the total length of the profile from the closest Bcl. Band-class designations were based on their relative position on the profile compared with the standard reference.

Taxonomic identity of Bcl was inferred by position-based comparison with a selection of previously generated clones that together represent all phylotypes identified in two cheetahs with phylogenetic clone library analysis [351]. This set of clones with known phylogenetic position was included as a taxonomic reference framework in DGGE to perform band position analysis in which a maximum deviation of 0.5% was allowed. Bands from potentially discriminating Bcl that could not be identified this way were excised from the DGGE gel, eluted into 40µl 1x TE buffer and heated for 10 min at 65°C. Subsequently, the DNA solutions were reamplified using the same V3-16S rRNA primers as those of the community PCR. Purity of excised gel fragments was checked by comigration of bands on DGGE gels with a narrower gradient and this procedure was repeated 2-3 times until a single band was obtained. Pure bands were analyzed on a new 35-70% DGGE gel adjacent to the original faecal sample to confirm their position in the Bcl. Excised bands were sequenced using an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems) by means of the Big Dye® XTerminator™ v.3.1. Cycle Sequencing and Purification Kit (Applied Biosystems) according to the protocol of the supplier. Likewise, identification results from band position analysis were also verified by band sequencing. Sequences were obtained using both forward (F357 without GC clamp) and reverse (R518) primers, and assembled using BioNumerics software. The web-based EzTaxon Server was used to allocate sequences to species based on 16S rRNA gene sequence similarities (http://www.ezbiocloud.net/eztaxon) [355].

DGGE data analysis

An initial exploration of similarities between DGGE profiles was performed by hierarchical cluster analysis with the unweighted pair group method with arithmetic mean (UPGMA) and using the Pearson correlation coefficient and Dice's coefficient. Student's t-test and Kruskal-Wallis test were used in SPSS v.21.0 for statistical comparison (α=0.05) of the variable range in pairwise similarities between the sample sets of PL and OV and between animals, respectively. In a statistically similar manner, also the community diversity was compared using DGGE band number (species richness, S), Shannon Diversity Index (H') and evenness ($E= H'/H'_{max}$, where $H'_{max}= \ln S$) as standard indices.

To assess the bacterial community stability, multiple approaches were used. First, faecal samples were binned into 15 increasing time intervals (i.e. 2 months, 4 months, 6 months, … up to 30 months between sample collection) after which the average Dice similarity coefficient across all possible sample pairs was determined in each bin. The temporal stability of the predominant faecal microbiota was then monitored by calculating the time in months between the sample dates as well as the fraction of shared Bcl between them as measured by the Dice coefficient. Next, the species richness S was plotted against time. In another approach, the evolution of the DGGE profile similarities between consecutive time points, *i.e.* the rate of change (Δt), was determined for each animal with moving window analysis, which was previously demonstrated to be a valuable tool for monitoring microbial community dynamics [356]. For this purpose, a matrix of similarities between the densitometric curves extracted from all DGGE patterns per animal was calculated based on the Pearson correlation coefficient. Based on the equation %change= 100-%similarity, similarity values were converted to %change values which were then plotted in a time frame of consecutive sampling points for moving window analysis. The rate of change value (Δt) was calculated as the average of the respective moving window curve data points and indicates the relative stability of the predominant microbiota within a specific animal over the study period. The higher the changes between the DGGE profiles of two consecutive sampling points, the higher the corresponding moving window curve data point will be, hence the higher the Δt values.

Potentially discriminating band-classes were identified by linear discriminant analysis on the total DGGE dataset with BioNumerics. In addition, quantitative information derived from relative band intensities was exported as a data matrix and exported to SPSS v21.0 for statistical analysis. Non-parametric Mann-Whitney U tests (α=0.05) were performed to substantiate the potential discriminating band-classes in DGGE fingerprint profiles of cheetahs grouped per zoo sample set or per animal. *P*-values were corrected for multiple-testing error by using the Benjamini-Hochberg method [357].

Real-time PCR

The choice of the four 16S rRNA gene-targeted group-specific real-time PCR assays (**TABLE S5.1**) was based on the high abundance of *Clostridium* clusters I, XI and XIVa, and near absence of *Bifidobacteriaceae* previously observed in our clone library analysis of two cheetah samples [351]. In addition, also the Firmicutes to Bacteroidetes ratio was determined. Real-time PCR amplification and detection were performed in a Lightcycler®480 Real-Time PCR System Instrument II (Roche). Each reaction mixture (20 µl) was composed of 10 µl 2x SensiMix SYBR® No-ROX kit (Bioline), 0.3 µl of each specific primer (10 mM), 6.4 µl milliQ water and 3 µl of stock or 10x diluted template DNA. All reactions were run in triplicate and the fluorescent product was detected in the last step of each cycle. Following amplification, melting temperature analysis of PCR products was performed to determine the specificity of the PCR. The melting curves were obtained by slow heating at 0.2°C/s increments from 65°C to 97°C, with continuous fluorescence collection. The relative amount of Firmicutes and Bacteroidetes 16S rRNA in each sample was normalized to the total amount of faecal bacteria by the comparative C_T method [358].

For quantification of *Clostridium* clusters I, XI, XIVa and bifidobacteria, fluorescent signals were plotted against group-specific external standard curves derived from 10-fold serial dilutions of taxonomic reference cultures with known concentrations. All obtained standard curves met the required standards of efficiency (R^2>0.99, 90%>E>115%) [359]. Results were transformed to log10 CFU/g wet weight faeces values.

Real-time PCR data analysis

Data obtained with real-time PCR assays were visualized in boxplots. Student's t-test and Kruskal-Wallis test (α=0.05) were applied to compare the Firmicutes/Bacteroidetes ratio and mean concentrations of *Clostridium* cluster I, XI, XIVa and *Bifidobacterium* spp. between zoo sample sets and animals respectively. Following correlation analysis (α=0.05) in SPSS, scatter plots were generated to mirror the association between target bacterial groups. Moving-window analysis was also carried out on absolute quantities (CFU/g) of *Clostridium* clusters.

5.3. Results

5.3.1. Variation and diversity of predominant bacteria from cheetah faecal samples

The 55 faecal samples collected bimonthly from 5 cheetahs over a 3-year period were initially compared by hierarchical UPGMA clustering of DGGE profiles using the Pearson and Dice coefficient. Neither types of analysis revealed a host-specific clustering. However, samples tended to cluster together per zoo when only the presence/absence of bands in the DGGE profiles were taken into account (**FIG 5.1**). Calculation of the mean %pairwise similarity from 378 pairwise combinations for PL and 351 for OV derived from Dice/UPGMA clustering revealed that DGGE profiles of OV samples were overall

significantly (two-tailed Student's t-test, $P<0.05$) less similar (62.90% ± 0.55) and thus more variable in composition compared to the samples from PL (68.36% ± 0.47). This is also depicted by the overall longer branch distances between samples of OV in the dendrogram (**FIG 5.1**). Similar differences were also found when comparing the mean %pairwise similarity values per animal which ranged from 59.95% similarity between profiles of NL10 to 71.55% similarity between profiles of B1. Comparison of the mean %pairwise similarity values between the 5 animals revealed a significant difference (Kruskal-Wallis Test, $P<0.05$) between B2 (65.88% ± 1.08) and NL10 (59.95% ± 1.49), and between B1 (71.55% ± 1.18) and all the other cheetahs (B2 [65.88% ± 1.08], NL9 [64.08% ± 1.97], NL10 [59.95% ± 1.49] and NL11 [65.35% ± 1.48]).

As a first assessment of the population diversity revealed by DGGE, richness (S), evenness (E) and the Shannon Diversity Index (H') were calculated for each sample. S values, expressed as the number of bands, ranged from 9 to 26 with an average of 15 bands per profile. Comparison of diversity indices identified significant differences in mean S and H' (two-tailed Student's t-test, $P<0.05$) between samples from PL ($S_{PL}=14 \pm 1$; $H'_{PL}=1.01 \pm 0.02$) and from OV ($S_{OV}=16 \pm 1$; $H'_{OV}=1.07 \pm 0.03$). Among the 5 animals, no significant differences were found in mean richness ($S_{B1}=14$, $S_{B2}=14$, $S_{NL9}=16$, $S_{NL10}=16$, $S_{NL11}=17$) and mean Shannon Diversity Index values ($H'_{B1}=1.02$, $H'_{B2}=1.00$, $H'_{NL9}=1.06$, $H'_{NL10}=1.05$, $H'_{NL11}=1.11$) (Kruskal-Wallis test, $P_S=0.191$ and $P_{H'}=0.237$).

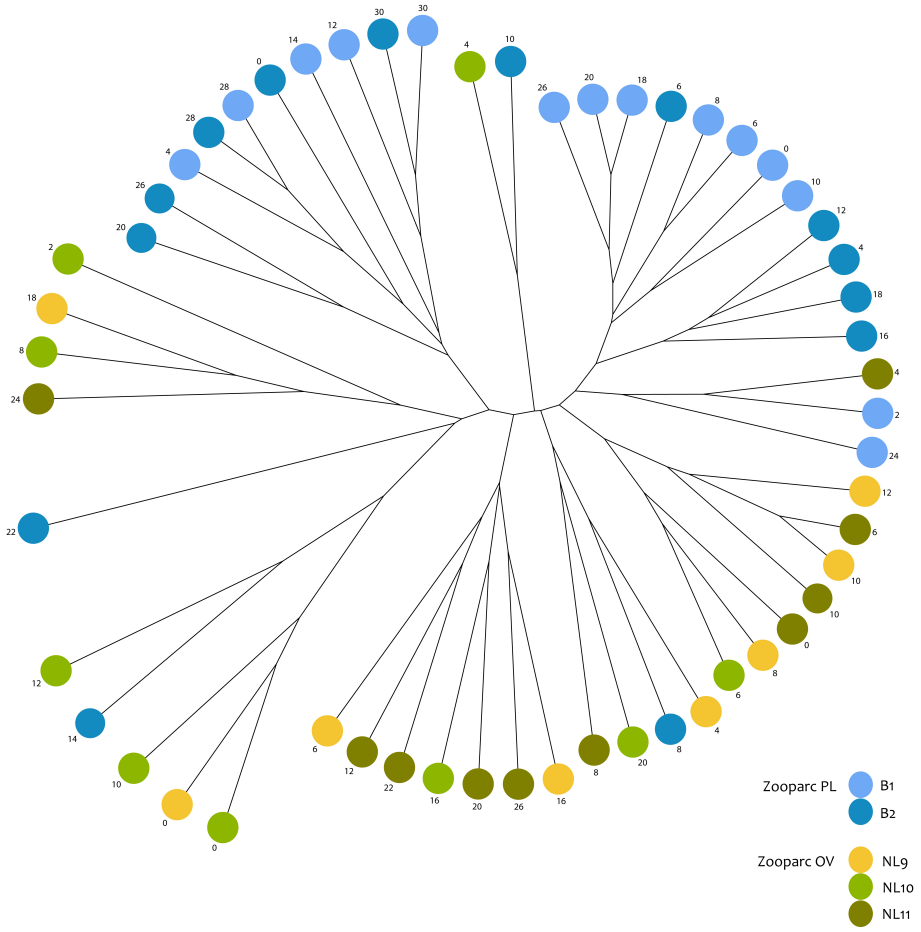


FIG 5.1 | Unrooted UPGMA dendrogram based on the Dice distances matrix depicting the similarities between DGGE profiles of faecal samples collected from 5 different captive cheetahs. Samples are represented by circles, coloured by animal and labelled with number of months passed since the start of sampling.

DGGE bands across all profiles were assigned to a total of 45 different Bcl of which 31 could be taxonomically assigned using combined DGGE band position and clone-library analysis. **TABLE S5.2** summarizes the distribution and taxonomic assignment of the Bcl and their relative abundances. Overall, 27 Bcl were common to all cheetahs, 8 were unique for samples from OV whereas none were unique for samples from PL. Twenty out of the 31 taxonomically identified Bcl were assigned to *Clostridium* clusters I (6 Bcl), XI (6 Bcl) and XIVa (8 Bcl), respectively. Linear discriminant analysis and statistical analysis of Bcl intensities identified 19 of the 45 Bcl to be potentially discriminating between the sample sets from PL and OV (Mann-Whitney U tests, $P<0.05$). Benjamini-Hochberg correction for multiple testing reduced this number to 9 discriminating Bcl. Six out of these 9 Bcl were assigned to a taxonomic group by band position analysis and represented members of *Clostridium* cluster XI (Bcl 78.65 and 80.93), *Clostridium* cluster I (Bcl 77.79), the *Lactobacillaceae/Enterococcaceae* group (Bcl 38.50), *Streptococcaceae* (Bcl 32.59) and *Erysipelotrichaceae* (Bcl 39.81). Sequencing of bands corresponding to the three unidentified Bcl 28.14, 72.37 and 81.76 revealed 16S rRNA gene sequence similarity with *Eubacteriaceae*, *Peptostreptococcaceae* and *Clostridiaceae*, respectively. The strongest discriminators between PL and OV ($P<0.01$) were Bcl 77.79, Bcl 39.81, Bcl 28.14 and Bcl 81.76 (all more prominent in OV) and Bcl 38.50 (more prominent in PL). Comparison of Bcl between animals showed no significant differences after Benjamini-Hochberg correction for multiple testing (Kruskal-Wallis Test, $P>0.05$) and revealed no discriminating Bcl between animals (**TABLE 5.1**).

TABLE 5.1 | Distribution of discriminating band-classes between sample sets of zoos PL and OV.

		ZOOPARC PL		ZOOPARC OV			
Taxonomic assignment at family or *Clostridium* cluster level[a]	**Bcl**	**Frequency (%; n=28)**	**median band intensity (min-max)**	**Frequency (%; n=27)**	**median band intensity (min-max)**	***P*-adjusted[b]**	**Closest type strain[a]**
Clostridium cluster I	77.79	10.7	0 (0-35.1)	51.9	31 (0-127)	<0.01	*Eubacterium multiforme* JCM 6484^T; *Clostridium sardiniense* DSM 2632^T
	81.76	10.7	0 (0-176.3)	59.3	181 (0-222.1)	<0.01	*Clostridium fallax ATCC 19400*^{T}
Clostridium cluster XI	28.14	14.3	0 (0-18.90)	55.6	11 (0-78.9)	<0.01	*Eubacterium tenue* DSM 6191^T
	78.65	21.4	24.5 (0-86.7)	51.9	0 (0-56.8)	0.045	*Peptostreptococcus stomatis* W2278^T
	80.93	82.1	142.5 (0-226.9)	85.2	214 (0-240)	0.039	*Clostridium hiranonis* TO-931^T
Lactobacillaceae / Enterococcaceae	38.5	78.6	65.1 (0-220)	22.2	0 (0-191.5)	<0.01	*Enterococcus cecorum* ATCC 43198^T; *Lactobacillus sakei* DSM 20017^T
Streptococcaceae	32.59	50	8 (0-89)	11.1	0 (0-127.9)	0.026	*Lactococcus piscium* CCUG 32732^T
Erysipelotrichaceae	39.81	28.6	0 (0-137.6)	96.3	44 (0-160)	<0.01	*Turicibacter sanguinis* DSM 14220^T
Peptostreptococcaceae	72.37	0	0 (0-0)	29.6	0 (0-89.6)	0.015	*Peptostreptococcus stomatis W2278*^{T}

[a]Taxonomic assignment based on a clone library analysis from faecal samples of captive cheetahs or sequencing of excised bands for Bcl 28.14, 72.27 and 81.76

[b]*P*-values based on non-parametric Mann-Whitney U tests ($\alpha=0.05$)

5.3.2. Temporal stability of the predominant faecal microbiota of five cheetahs assessed by DGGE

Monitoring of shared Bcl across 15 increasing time intervals disclosed that the microbiota composition within each cheetah was relatively stable (averages between 60 and 71%), with 65.34 ± 2,06% of the same Bcl retrieved after 1 year (**FIG 5.2**). The number of Bcl shared between shorter sampling intervals was not significantly different compared to long intervals (Kruskal-Wallis test, P=0.974), which indicates the persistence of a core set of species over a prolonged period of time. To define the stability of a given Bcl in function of its relative abundance, the mean sample prevalence of Bcl shared by all animals (45.45 ± 4.08% of all samples) was compared to the prevalence of non-shared Bcl (14.24 ± 2.70% of all samples). This analysis revealed a significant difference (Mannheim-Whitney U test, P<0.01) between both, suggesting that the more stable members of the microbiota are also the most frequently occurring ones. The mean variation in number of bands (mean S_{var}), as a proxy for variation in richness, tended to be higher for animals from OV (mean S_{varNL9}= 5, mean $S_{varNL10}$= 6, $S_{varNL11}$= 5) than for animals from PL (mean S_{varB1}= 3, mean S_{varB2}= 3). The greatest S range over time was observed for NL10, which was mainly due to a single high increase in number of bands two months after the first sampling.

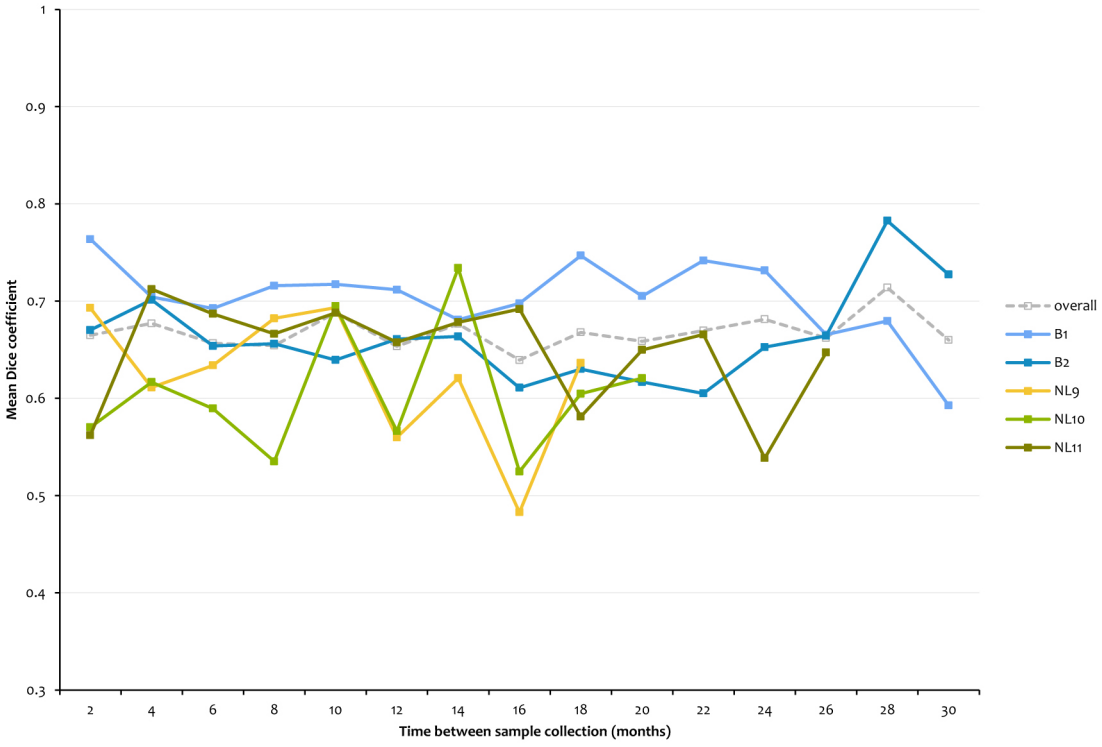


FIG 5.2 | The mean Dice coefficients between samples binned into sampling intervals and depicted per cheetah.

In addition to the presence or absence of bands, their relative abundance was also considered by calculating the rate of change values (Δt) between profiles for each animal. Moving window correlation analysis for DGGE fingerprint profiles showed the highest rate of change values for NL9 (Δt= 39.4%) (**FIG 5.3**) which could be mainly attributed to an increase in %change between consecutive samples taken at the 4th and 6th month of sampling and the 12th and 16th month of sampling. Such fluctuations were not observed for the other cheetahs over the 30 months period as evidenced by lower Δt ranges (23.3 - 25.6 %change) between consecutive sampling points.

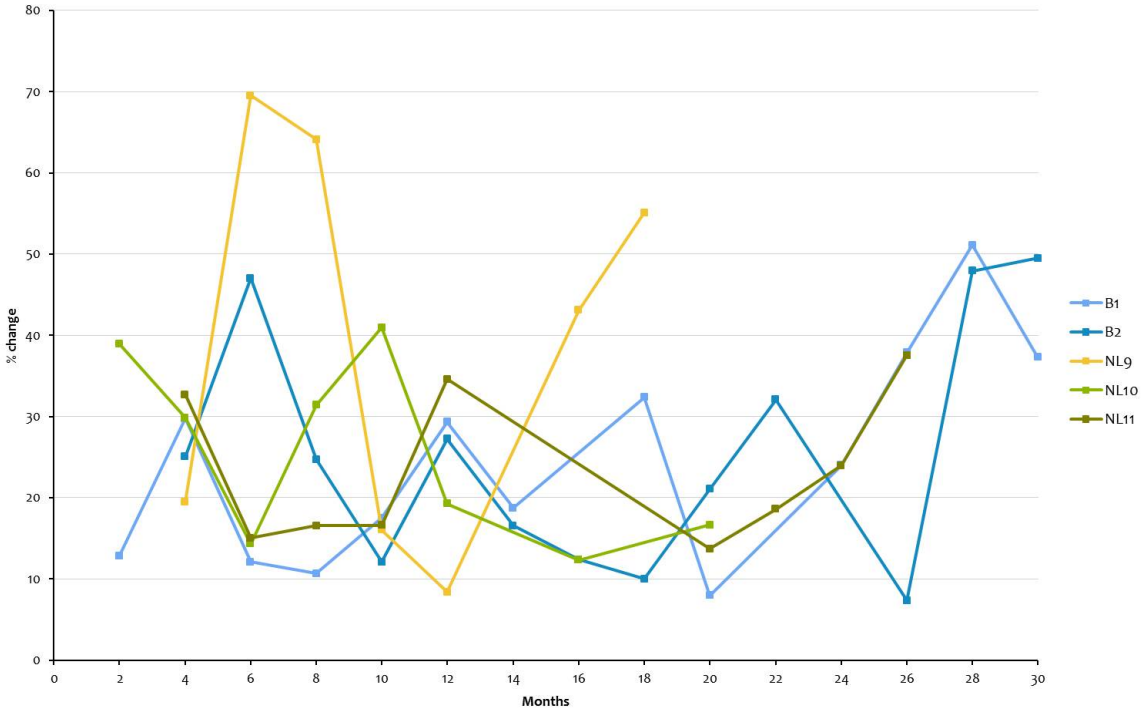


FIG 5.3 | Moving-window analysis and rate of change (Δt) value of consecutive DGGE profiles per animal. Variability between month x and month x-2 was calculated from a matrix of similarities for DGGE patterns, based on the Pearson correlation coefficient.

5.3.3. Real-time PCR quantification and temporal stability of *Clostridium* clusters I, XI and XIVa, bifidobacteria and F/B

Members of *Clostridium* clusters XI (mean= 7.50 ± 0.09 log10 CFU/g) and XIVa (mean= 9.19 ± 0.13 log10 CFU/g) were detected in quantifiable concentrations in all 55 samples, whereas members of *Clostridium* cluster I (mean= 8.51 ± 0.07 log10 CFU/g) could not be detected in two samples of NL10 and in one sample of NL11, B2 and NL9. In agreement with these results, the DGGE profiles of these samples did not contain Bcl that were assigned to *Clostridium* cluster I. Bifidobacteria were only detected in 23 samples (42% of total), with a mean concentration of 5.59 ± 0.19 log10 CFU/g. Boxplot analysis revealed a significantly higher concentration of *Bifidobacterium* spp. in samples from OV (6.02 ± 0.03 log10 CFU/g) compared to samples from PL (5.25 ± 0.18 log10 CFU/g) (two-tailed Student's t-test, $P<0.05$) (**FIG 5.4**). Between animals, however, quantitative ranges of the specific bacterial groups were not significantly different (Kruskal-Wallis Test, $P>0.05$).

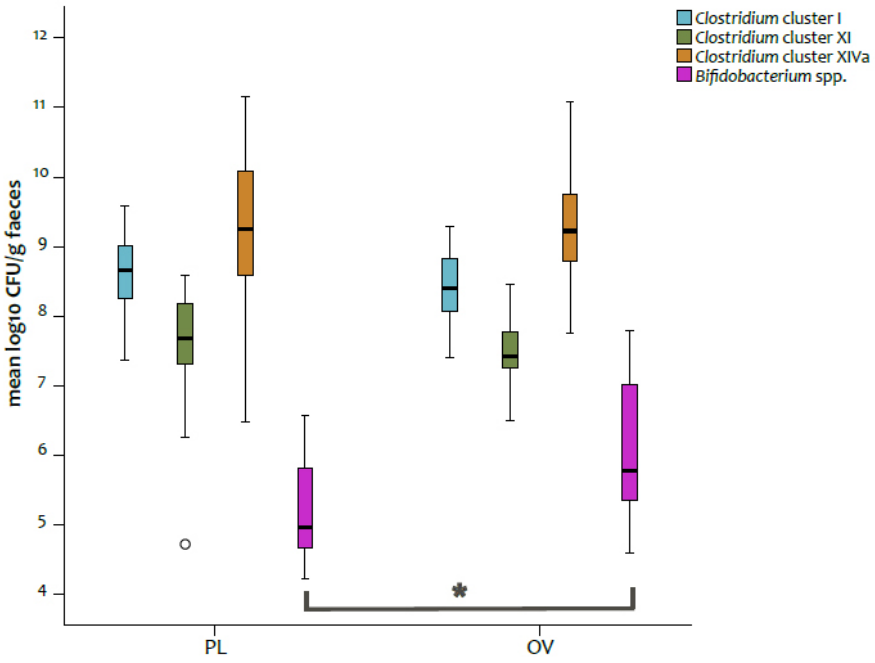


FIG 5.4 | Concentrations of *Clostridium* cluster I, XI, XIVa and Bifidobacterium spp. in faecal samples of 2 cheetahs from Zoo Parc Planckendael (PL) and 3 cheetahs from Zoo Parc Overloon (OV), assessed by real-time PCR. Boxplots show median, interquartile range, sample minimum and maximum. The open circle indicates an outlier value for animal B2. *$P<0.05$, two-tailed Student's t-test

Correlation analysis, expressed as scatter plots, indicated a positive correlation between the relative concentrations of *Clostridium* clusters I, XI and XIVa ($P<0.05$; $P<0.01$) (**FIG 5.5**). No significant correlation was found between the relative concentrations of *Bifidobacterium* spp. and *Clostridium* clusters I ($P= 0.468$), XI ($P= 0.483$), and XIVa ($P= 0.711$), respectively.

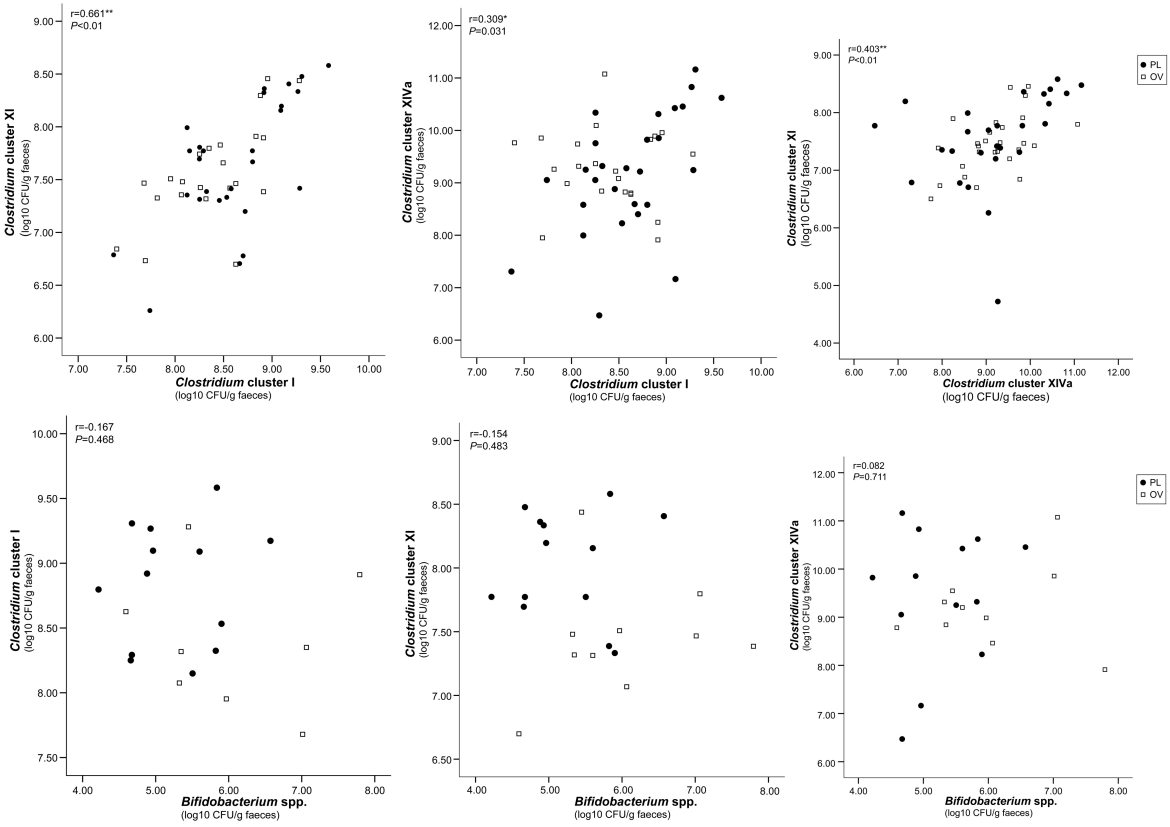
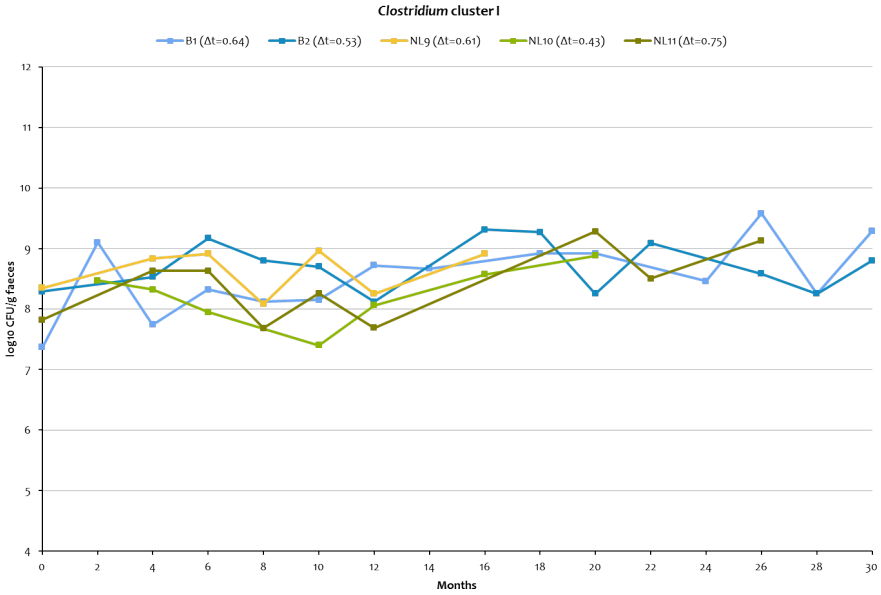
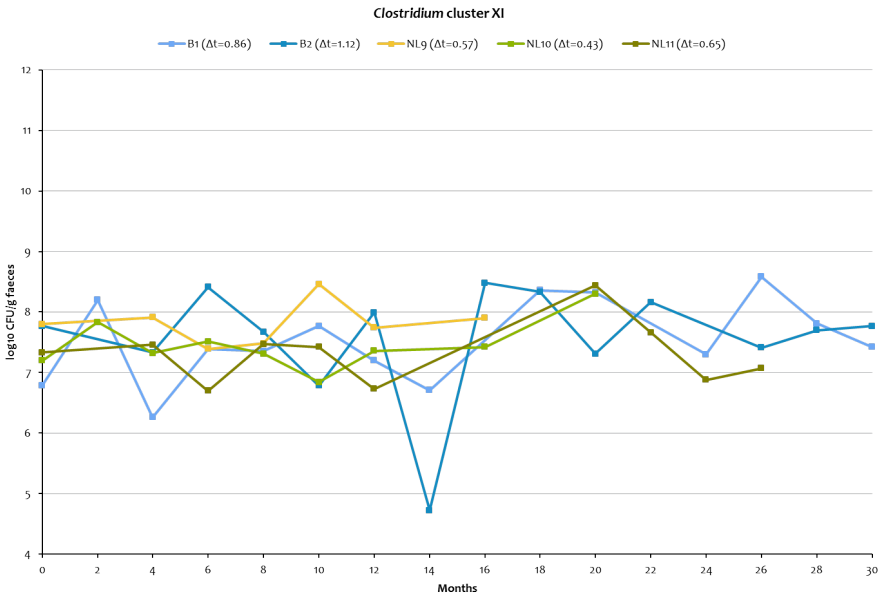


FIG 5.5 | Scatter plots displaying the correlation between log10 concentrations of bacterial groups quantified with real-time PCR in captive cheetah's faecal samples. r=Pearson correlation coefficient, *correlation significant at the 0.05 level, **correlation significant at the 0.01 level

The temporal variation of these concentrations for each animal is shown in **FIG 5.6** and was not significantly different between animals (Kruskal-Wallis Test, *P*>0.05). On average, overall concentrations of *Clostridium* cluster I, XI and XIVa varied over a 30 month time period with only 0.59, 0.72, and 0.87 log10 CFU/g, respectively. During the disease period of animal NL9, an increase (+0.9 log10 CFU/g) in members of *Clostridium* cluster I and a decrease (-1.9 log10 CFU/g) in *Clostridium* cluster XIVa members were observed. For the ratios of Firmicutes to Bacteroidetes, no significant differences were found between sample sets from PL and OV or between animals. However, for animal NL10 Firmicutes to Bacteroidetes ratios displayed a relatively broader range, from 0.5/0.003 to 0.8/2.2e-09.





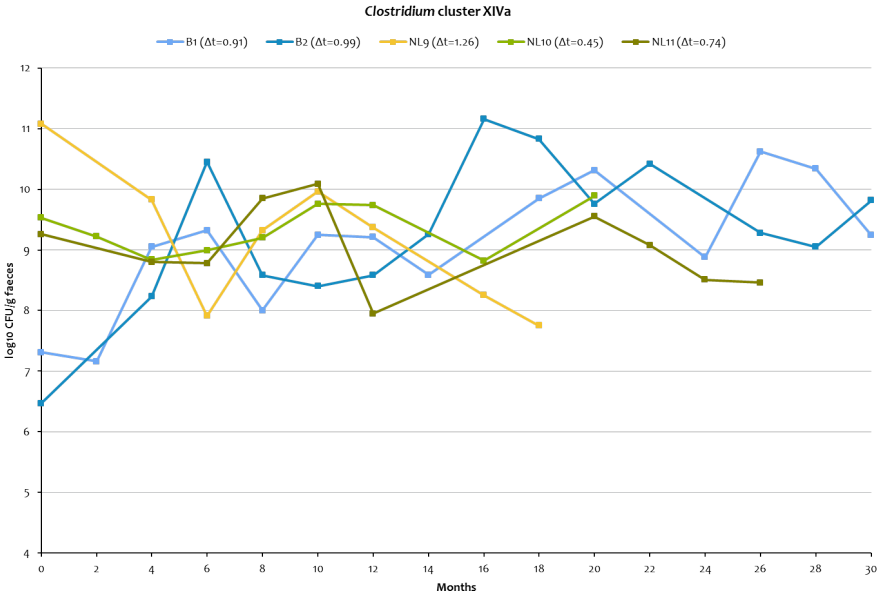


FIG 5.6 | Temporal variation of *Clostridium* clusters I, XI and XIVa in faecal samples of 5 captive cheetahs, assessed by real-time PCR. Rate of change (Δt) value of consecutive DGGE profiles calculated per animal

5.4. Discussion

The few investigations performed so far on the diversity of the cheetah gut microbiota were limited in scope as they were based on single time point samples from a total of four captive cheetahs using different taxonomic methods [86,351]. Although these studies were instrumental in providing a first cross-sectional insight in the gut microbiota composition of cheetahs under human care, it remained unclear to what extent this intestinal ecosystem exhibits temporal stability over a prolonged time period. In the present study, we monitored the predominant microbiota compositions and the quantitative abundance of selected microbiota members in five cheetahs housed in two geographically separated zoos during a bimonthly sampling campaign over a period of three years.

Our combined approach of DGGE and real-time PCR revealed that the five animals under study exhibit an overall stable faecal bacterial composition, and the inclusion of additional animals in follow-up investigations will undoubtedly help to reveal if this finding is a characteristic across the entire animal species. We found that 67% of the detected Bcl in DGGE profiles was retained over a period of 30 months, with the majority of the recurring Bcl corresponding to bacterial core phylogroups. Also at the phylum level, minor fluctuations (±2.1%) were observed in the Firmicutes/Bacteroidetes ratio. The average life span of cheetahs is 10 to 12 years, though cheetahs under human care can live as long as 20 years [360]. When taking into account the age of each animal, this means that the observed temporal stability in the study's time frame covered four cheetahs during early aging and one cheetah (NL11) in late adulthood. Our insights for cheetahs, as well as data from long-term monitoring studies of lab mice and wild chimpanzees [361,362], seem to indicate that gut microbiota stability is a general feature in mammals during adulthood. After a highly unstable early stage of microbial colonization in the mammalian GI tract during the neonatal period, complex interactions between host and resident

bacteria lead towards a stable adult-like microbiota. These changes are shared among different mammalian species but differ in timing and some species-specific developmental events [68,363]. Decreased stability and shifts in diversity have been observed with advancing age in humans [364–366], and similar microbial changes were also observed in dogs [367,368]. The fact that long-term stability of gut bacteria communities is maintained during adulthood supports the concept of an ecosystem with a core community that operates in a state of homeostasis, and to a certain degree exhibits resilience to perturbations [88].

With a presence of ≥80% in the samples collected per animal over a time span of 30 months, *Clostridium* clusters XI and XIVa appear to dominate the phylogenetic core of the faecal microbiota in captive cheetahs. Together with *Clostridium* cluster I, represented in ≥50% of the samples per animal, these groups are considered relatively stable microbiota members, as evidenced by the limited fluctuations revealed by the respective qPCR assays. Monitoring studies (2-6 months) in other carnivores such as captive and wild grizzly bears also indicated that in the majority of samples the relative rank of *Clostridium* clusters I, XI and XIV determined by specific qPCR assays remained constant [265,268]. Furthermore, bacterial numbers reported for the aforementioned clusters in these studies were in the same order of magnitude (7-8 log10 CFU/g) as those obtained for captive cheetahs in the present study. For the older cheetah NL9, *Clostridium* cluster XIVa numbers were more variable, which might not only be linked to the disease period but also to an overall higher variability of this cluster upon aging, as has also been shown in humans [364].

This study confirms that in carnivores a positive correlation exists between abundances of *Clostridium* cluster I and XI [332,351]. *Clostridium* cluster I includes proteolytic and fibrolytic species, but also harbours toxinogenic members such as *C. perfringens* [369]. The latter species was detected in almost all faecal samples of the five cheetahs included in this study, which corroborates earlier observations suggesting that it is a common inhabitant of the cheetah's intestinal tract [351]. *C. perfringens* has also been detected regularly in faeces of other carnivores [265,267,343,370,371], including domestic cats [282] and wild exotic felids [370]. Animals included in these studies were all considered healthy. In contrast, a number of case reports have described neurotoxicity, hemorrhagic enterocolitis and death in captive tigers, lions and cheetahs due to *C. perfringens* infections and toxins [45,372,373]. In non-infected animals, *C. perfringens* should probably be considered as a common commensal that is positively correlated to the high protein content of the carnivore's diet [265,314]. However, further studies are needed to clarify the role of different genotypes of *C. perfringens* as causal agents of enterocolitis and diarrhoea in carnivore species [370].

Lactobacillaceae are also considered to be part of the core gut microbiome of carnivores [344]. This was confirmed in the current study by their presence in ≥50% of the samples of all five cheetahs throughout the 30 months sampling period, suggesting *Lactobacillaceae* are stable members of the cheetah's GI core microbiota. In contrast, representatives of *Carnobacteriaceae*, *Erysipelotrichaceae* and

Enterobacteriaceae comprised most of the transient members of the cheetah's gut microbiota. Members of the latter family have been shown to be transiently excreted in faeces of domestic cats [287]. *Bifidobacterium* spp. are considered common members of the gut microbiota in domestic cats [272], but in this study they were detected in less than half of the samples suggesting that bifidobacteria take no part in the core composition of the intestinal ecosystem in captive cheetahs.

A valid interpretation of temporal stability data benefits from additional cross-sectional analyses that may potentially link specific microbiota variations to zoo environment or animal characteristics. Pooled data analysis of overall species richness, the number of unique phylotypes derived from DGGE BcI analysis and median BcI intensities indicated no significant differences at animal level, but indicated a number of differences between PL and OV sample sets that were mostly the result of variation in proportional composition at the phylotype level. At phylum level, however, the mean ratios of Firmicutes to Bacteroidetes were comparable for both sample sets throughout the study. One of the strongest discriminators that separated the two sample sets was the significantly higher prevalence of *Erysipelotrichaceae* in OV samples. In a previous study in mice, increased levels of *Erysipelotrichaceae* were linked to an increased dietary fat content [374]. Possibly, a higher dietary fat intake resulting from the more regular intake of whole prey and prey organs [375,376] by the cheetahs in OV may explain the association with higher *Erysipelotrichaceae* numbers.

No significant differences were observed upon comparison of the five cheetah's individual microbial profiles. Likely, the minor variations observed in the predominant microbiota composition of each cheetah between consecutive sampling points reflect changes imposed by technical sensitivity and specificity and an unknown combination of intrinsic factors such as genetics, life history, diet, human and other animal exposures, and health status [377–379]. Of these, disease manifestation and subsequent therapy may be amongst the most powerful disruptors of microbiota stability as exemplified by animal NL9 that suffered from intermediate episodes of vomiting and diarrhoea during the monitoring period. This cheetah was treated with Zitac® vet tablets, containing the gastric acid production inhibitor cimetidine and the amoxicillin-clavulanate antibiotic Clavubactin®. During that disease period, an increase in members of *Clostridium* cluster I and a decrease in *Clostridium* cluster XIVa members were observed. Upon recovery, both clusters normalized to their initial concentrations as determined before the onset of symptoms. These changes in two microbial key groups of the cheetah's intestinal ecosystem might reflect a state of dysbiosis resulting from compromised health, but may also point to a temporary disturbance upon administration of antibiotics or a combination thereof. For instance, a significant reduction of members of *Clostridium* cluster XIVa has also been observed in the faecal microbiota of a gnotobiotic mouse model following a 7 day amoxicillin-clavulanate administration [380]. Additionally, DGGE profiles for samples from animal NL9 were characterized by a temporary decrease in species richness and change in intensity of bands, which was reflected in a higher rate of change value upon moving-window analysis. In this particular case, it appears that the relative

proportions rather than the composition of the animal's core microbiota groups were altered. It should be kept in mind, however, that the number and intensity of bands in a DGGE gel do not necessarily give an accurate picture of the microbial community due to multicopy operon heterogeneity of the 16S rRNA gene and the limited phylogenetic resolution of this technique [212,381,382].

Despite the limitations in detection level and taxonomic resolution, the phylogenetic information extracted from the integrated DGGE clone library approach allowed us to identify a set of major bacterial lineages that may constitute the core microbiome of cheetahs under human care. Mammalian microbiota profiles tend to cluster according to the classification of their hosts into herbivores, omnivores and carnivores, which suggests that gut physiology and diet are powerful predictors of faecal microbiota composition [86,311]. However, the fact that microbiomes of conspecifics tend to resemble one another at a broader (e.g. phylum) taxonomic level does not imply that they also host similar phylotypes. This is exemplified in a recent oligotyping-based inventory of faecal bacterial communities in single samples from 68 free-ranging Namibian cheetahs [264] which revealed marked differences in some dominant phyla proportions compared to captive cheetahs. Overall, free-ranging cheetahs exhibited lower proportions of Firmicutes and higher proportions of Fusobacteria and Bacteroidetes. At the genus level, however, *Clostridium* spp. and *Blautia* spp. prevailed which further underpins their role as core microbiome members of the cheetah gut. In addition, members of the *Coriobacteriaceae* which are considered frequent residents of the feline gut [272] appeared to be enriched in free-ranging cheetahs [264], whereas this group made up only a small proportion of the clone libraries from two captive cheetahs [351]. Although these observations suggest differences in core microbial pattern between free-ranging cheetahs and captive cheetahs, microbiome studies including both zoo and wild animal populations are warranted to fully depict gut microbial diversity and stability in the strict carnivorous cheetah.

5.5. Conclusions

The findings of this long-term monitoring study evidence that captive cheetahs host an overall stable faecal microbiota, even beyond intestinal community variations between zoo sample sets and to a lesser extent, between animals. The core of this microbiota appears to be mainly dominated by members of *Clostridium* clusters I, XI and XIVa and *Lactobacillaceae*, most of which were maintained at stable levels throughout the monitoring period. It has been previously suggested that yearly examination of a faecal sample would be sufficient to monitor changes in the intestinal microbiota composition and stability and could promote disease prevention [87]. Our finding of significant disturbances in relative proportions of key microbial groups upon disease and/or therapy in one specific cheetah seems to support this idea. For wildlife species under human care, health indices other than current faecal steroid monitoring and faecal parasite and pathogen loads may thus also prove to be useful in health surveillance.

SUPPLEMENTARY DATA

TABLE S5.1 | 16S rRNA gene-targeted group-specific primers, taxonomic reference strains and amplification programs used in real-time assays.

Target bacterial group	Primer	Sequence (5'-3')	Taxonomic reference strains used for external standard curves	PCR program[a]	Adapted from
***Clostridium* cluster I**	CI-F CI-R	ATGCAAGTCGAGCGAKG TATGCGGTATTAATCTYCCTTT	*Clostridium perfringens* LMG 11264[b]	40x (95°C-15s; 55°C-20s; 72°C-30s)	[383]
***Clostridium* cluster XI**	CXI-F CXI-R	ACGCTACTTGAGGAGGA GAGCCGTAGCCTTTCACT	*Clostridium bifermentans* LMG 3029[b]	45x (95°C-20s; 58°C-30s; 72°C-45s)	[196]
***Clostridium* cluster XIVa**	Ccocc-F Ccocc-R	CGGTACCTGACTAAGAAGC AGTTTYATTCTTGCGAACG	*Ruminococcus gnavus* LMG 27713[c]	40x (95°C-15s; 55°C-20s; 72°C-30s; 80°C-30s)	[383]
Bifidobacterium	g-Bifid-F g-Bifid-R	CTCCTGGAAACGGGTGG GGTGTTCTTCCCGATATCTACA	*Bifidobacterium adolescentis* LMG 10502[d]	40x (94°C-20s; 55°C-20s; 72°C-50s)	[384]
Firmicutes	Firm934F Firm1060R	GGAGYATGTGGTTTAATTCGAAGCA AGCTGACGACAACCATGCAC	n/a	45x (95°C-10s; 60°C-30s; 72°C-1)	[194,316,317]
Bacteroidetes	Bact934F Bact1060R	GGARCATGTGGTTTAATTCGATGAT AGCTGACGACAACCATGCAG	n/a		
Total bacteria	Eub338F Eub518R	ACTCCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	n/a		

n/a = not applicable

[a] All amplification programs are preceeded by initial denaturation at 95°C for 10 min

[b] Grown on reinforced clostridial agar medium (CM0151, Oxoid)

[c] Grown on BBL™ Brain Heart Infusion medium, supplemented with 5g/L yeast extract (LP0021, Oxoid) and 5 mg/L hemin (H9039-1G, Sigma-Aldrich)

[d] Grown on modified Columbia agar [385]

TABLE S5.2 | Combined band-class and clone library analysis for 55 DGGE fingerprint profiles from faecal samples from 5 captive cheetahs.

Taxonomic assignment at family or *Clostridium* cluster level[a]	Band-class	Distribution							Closest type strain[a]
		Number of animals (n=5)	% of samples (n=55)	% of samples per animal					
				B1 (n=14)	B2 (n=14)	NL9 (n=8)	NL10 (n=9)	NL11 (n=10)	
Clostridium cluster I	64.99	*****	18.18	7	21	13	11	40	*Clostridium colicanis* DSM 2632^T
	67.39	*****	78.18	86	79	75	44	100	*Clostridium perfringens* ATCC 13124^T
	68.15	*****	69.09	79	79	75	33	70	*Clostridium perfringens* ATCC 13124^T
	70.79	*****	36.36	43	57	38	11	20	*Clostridium fallax* ATCC 19400^T
	76.09	*****	21.82	21	7	25	11	50	*Eubacterium multiforme* JCM 6484^T
	77.79	****	30.91	0	21	63	33	60	*Eubacterium multiforme* JCM 6484^T; *Clostridium sardiniense* DSM 2632^T
Clostridium cluster XI	73.47	***	9.09	0	0	25	11	20	*Clostridium glycolicum* DSM 1288^T
	74.60	*****	25.45	21	7	38	22	50	*Peptostreptococcus anaerobius* NCTC 11460^T
	75.34	*****	23.64	21	21	25	22	30	*Clostridium glycolicum* DSM 1288^T
	78.65	*****	41.82	71	50	13	22	30	*Peptostreptococcus stomatis* W2278^T
	80.11	****	16.36	14	36	13	0	10	*Clostridium hiranonis* TO-931^T
	80.93	*****	**83.64**	**79**	**86**	**100**	**89**	**70**	***Clostridium hiranonis* TO-931^T**
Clostridium cluster XIVa	47.13	*****	16.36	21	7	25	11	20	*Ruminococcus torques* ATCC 27756^T
	47.69	*****	45.45	50	21	50	44	70	*Blautia hansenii* JCM 14655^T
	48.97	*****	47.27	36	57	50	56	40	*Blautia coccoides* JCM 1395^T; *Blautia glucerasei* HFTH-1^T
	50.12	*****	69.09	71	79	50	67	70	*Blautia hansenii* JCM 14655^T; *Blautia glucerasei* HFTH-1^T
	50.60	*****	41.82	50	36	50	67	70	*Clostridium boltae* DSM 15670^T
	53.61	*****	56.36	71	50	50	56	50	*Blautia glucerasei* HFTH-1^T
	54.43	*****	**94.55**	**93**	**100**	**100**	**89**	**90**	***Ruminococcus gnavus* ATCC 29149^T**
	57.31	*****	54.55	57	64	75	33	40	*Coprococcus comes* ATCC 27758^T
Lactobacillaceae	37.19	*****	29.09	7	21	50	56	30	*Lactobacillus sakei* DSM 20017^T
	61.53	**	3.64	0	0	13	11	0	*Lactobacillus mucosae* CCUG 32732^T
Lactobacillaceae / Enterococcaceae	38.50	*****	50.91	86	71	13	11	40	*Enterococcus cecorum* ATCC 43198^T; *Lactobacillus sakei* DSM 20017^T
Enterococcaceae	35.21	*****	49.09	50	21	50	89	50	*Enterococcus hirae* DSM 20160^T
	43.67	*****	60.00	79	64	25	78	40	*Enterococcus faecalis* JCM 5803^T
Streptococcaceae	32.59	****	30.91	64	36	0	11	20	*Lactococcus piscium* CCUG 32732^T
Erysipelotrichaceae	39.81	*****	61.82	43	14	100	100	90	*Turicibacter sanguinis* DSM 14220^T

Taxonomic assignment at family or *Clostridium* cluster level[a]	**Band-class**	**Distribution**							**Closest type strain**[a]
		Number of animals (n=5)	% of samples (n=55)	% of samples per animal					
				B1 (n=14)	B2 (n=14)	NL9 (n=8)	NL10 (n=9)	NL11 (n=10)	
Carnobacteriaceae	42.45	****	21.82	14	36	0	22	30	*Carnobacterium divergens* DSM 20623^T
Peptococcaceae	51.96	*	1.82	0	0	0	11	0	*Desulfonispora thiosulfatigenes* DSM 11270^T
Enterobacteriaceae	58.50	****	43.64	29	64	0	89	30	*Shigella flexneri* ATCC 29903^T
Lachnospiraceae	65.88	**	9.09	0	0	0	11	40	*Cellulosilyticum ruminicola* H1^T
No match	25.61	*	3.64	0	0	25	0	0	-
	27.24	***	9.09	0	0	25	22	10	
	28.14	*****	34.55	7	21	63	56	50	
	29.33	*****	36.36	36	21	38	44	50	
	30.81	****	12.73	7	0	13	22	30	
	34.77	****	16.36	7	14	38	0	30	
	41.28	*****	41.82	43	57	25	33	40	
	45.17	****	18.18	7	36	0	22	20	
	55.77	**	3.64	7	0	13	0	0	
	63.89	***	7.27	0	0	13	11	20	
	68.79	*****	10.91	7	7	13	22	10	
	72.37	***	14.55	0	0	25	11	50	
	81.76	*****	34.55	7	14	63	67	50	
	82.76	**	3.64	0	7	0	0	10	

[a]Based on a clone library analysis from faecal samples of captive cheetahs [351]

- = no matching clones; ***** = number of animals

Discriminating Bcl between Zoo Parc Planckendael (PL) and Zoo Parc Overloon (OV) are marked in grey and stable Bcl over the 3-year sampling period (>80% of the samples per animal) are marked in bold

Submitted to PloS One:

Becker AAMJ, Van de Wiele T, Jauregui R, Vilchez-Vargas R, Pieper DH, Hesta M, Janssens GPJ, Huys G. The faecal microbiota of captive cheetahs: concordance of global phylogenetic diversity and temporal stability patterns derived from conventional molecular tools versus next-generation sequencing.

6 THE FAECAL MICROBIOTA OF CAPTIVE CHEETAHS: CONCORDANCE OF GLOBAL PHYLOGENETIC DIVERSITY AND TEMPORAL STABILITY PATTERNS DERIVED FROM CONVENTIONAL MOLECULAR TOOLS VERSUS NEXT-GENERATION SEQUENCING

The rapid succession of next-generation sequencing (NGS) technologies not only changed the experimental landscape of microbial ecology, but also questions the role of traditional molecular techniques in contemporary studies of complex bacteria communities. Here, we present a case study in the field of animal gut ecology on the added value of Illumina MiSeq sequencing to conventional molecular tools. Previously, we have evaluated the diversity and dynamics of the faecal microbiota of captive cheetahs (*Acinonyx jubatus*) using an integrated 16S rRNA gene DGGE-clone library approach in combination with a series of real-time PCR assays. In the present study, the methodological portfolio to investigate the same samples was expanded with the Illumina MiSeq platform. Illumina sequencing targeting the V5-V6 region of the 16S rRNA gene yielded 1 550 961 bacterial 16S rRNA gene reads from 25 previously characterized samples derived from five captive cheetahs housed at two different zoological parks. New Illumina data and existing DGGE-clone library results both identified representatives of *Clostridium* clusters I, XI and XIVa as prominent members of the phylogenetic core of the cheetah's faecal microbiota. In addition, Illumina MiSeq revealed 3 orders and 9 families not reported in previous studies but these represented a very marginal share of the total reads (0.1 and 0.43%, respectively). Compared to the preceding DGGE and real-time PCR results, the Illumina data set generated a similar shortlist of variables explaining bacterial community changes between zoo sample sets and between animals. In the temporal stability assessment, however, the NGS approach revealed subtle temporal variations in low-abundant taxa that were previously unnoticed. In our opinion, conventional molecular tools remain a valuable starting point for exploratory characterizations of complex microbial ecosystems such as the faecal microbiota of captive cheetahs. While contemporary sequencing methods such as Illumina MiSeq do not seem to contradict the global results obtained with these aforementioned approaches, they clearly add a more in-depth exploration of community composition and dynamics into the rare biosphere.

6.1. Background

Over the last decade, our view on microbial ecosystem landscapes has been revolutionized by the emerging use of next-generation sequencing (NGS) technologies. Especially the Illumina platform has provided a previously unseen capacity to interrogate the diversity of highly complex microbial ecosystems such as the human gut in a cost-effective and high-throughput manner [102,386], while still maintaining sufficient sensitivity to detect even rare taxa [387–389]. At a rapid pace, NGS technologies have become one of the standard approaches in contemporary microbial ecological studies and appear to have surpassed more traditional methods based on Sanger sequencing and community fingerprinting [213,387].

While novel applications of NGS technologies continue to challenge downstream data handling and analysis [390–394], traditional molecular techniques find themselves being reoriented in the toolbox of the microbial ecologist. In fact, for a part of the scientific community some of these seemingly surpassed technologies are still more affordable and accessible compared to NGS and offer sufficient potential to test the hypothesis in question [172]. For instance, community fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP) and automated ribosomal intergenic spacer analysis (ARISA) are still considered valid approaches to extract taxonomic information and/or provide diversity estimates in a rapid and relatively inexpensive way [212,213]. In recent years, several studies in fact employed an integrated approach in which NGS was combined with phylogenetic clone libraries and/or community profiling to uncover bacterial diversity in a wide range of environments [395–400]. This trend towards the complementary use of multiple molecular techniques seems to be triggered by various operational parameters such as flexibility, sensitivity, speed of analyses, complexity of data processing, and potential downstream applications. Several of these one-to-one comparisons between NGS and community profiling methods such as DGGE have clearly revealed that the former usually provides a deeper and more complete insight in sample diversity [397–400]. For those ecosystems and environments that have so far mainly been studied with non-NGS based methods, it is thus possible that current knowledge on microbial diversity is restricted to predominant community members and may overlook the rare biosphere and its role in ecosystem functioning.

Previously, we have provided a first-ever characterization of the intestinal ecosystem diversity and stability of the strictly carnivorous cheetah (*Acinonyx jubatus*) in captivity. The methodological strategy for that work was based on the analysis of faecal samples by using an integrated 16S ribosomal RNA (rRNA) gene DGGE-clone library approach in combination with a series of real-time PCR assays [351,401]. The present study aimed to evaluate to what extent additional information about the composition and the dynamics of the cheetah faecal microbiota can be obtained by using paired-end Illumina MiSeq sequencing targeting the V5-V6 hypervariable region of the 16S ribosomal RNA (rRNA) gene. For this purpose, existing DNA extracts from aforementioned studies were used in three comparative setups.

First, the correspondence between the faecal microbial diversity of two captive cheetahs either revealed by 16S rRNA phylogenetic clone libraries [351] or new Illumina MiSeq data was assessed. Second, the temporal stability profiles of microbial communities over a 3-year period in five captive cheetahs obtained from Illumina sequencing were compared to existing data from DGGE community fingerprinting and real-time PCR assays [401]. Finally, the datasets obtained from two different zoos were mutually compared using the aforementioned conventional molecular techniques and Illumina MiSeq.

6.2. Material & Methods

Ethics statement

This study was conducted non-invasively, without animal handling or any change in daily management and housing conditions of the animals. The cheetahs are housed according to the Minimum Standards for the Accommodation and Care of Animals in Zoos and Aquaria and the EAZA Code of Practice.
Permission for faecal sampling was obtained from the staff veterinarians and mammal curators

Study population, sample collection and DNA isolation

For this study a selection of existing samples from a previous sampling campaign were used. Over a two-year period, five adult cheetahs housed at two institutions that are full members of the European Association of Zoos and Aquaria (EAZA, http://www.eaza.net/membership) were bimonthly monitored for 12h to collect animal-specific faecal samples immediately upon defaecation. This way, a total of 55 fresh faecal samples (100-200 g/animal) were collected and previously analysed to assess the predominant gut microbial diversity and stability. Animal records and medical history, diet, housing and management were all well documented [401]. Zoo Parc Planckendael (PL, Belgium) housed cheetahs B1 and B2 from which 7 samples covering a two-year sampling period were included for each animal. Two of the samples included (sample B1_2 and B2_2) were also previously used to construct phylogenetic clone libraries [351]. Zoo Parc Overloon (OV, the Netherlands) housed cheetahs NL9, NL10 and NL11 from which 4 samples were included for NL10 and NL11 and 3 samples for NL9.
The protocol of Pitcher and co-workers [315] was used in a modified version [402] to extract total bacterial DNA from the faecal samples. DNA size and integrity were assessed on 1% agarose electrophoresis gels stained with ethidium bromide. DNA yields and purity were determined by measurements at 234, 260 and 280 nm using a Nanodrop 2000 spectrophotometer (Thermo, Scientific).

Illumina library generation and bioinformatic analysis

To identify the bacterial community, Illumina MiSeq sequencing targeting the V5-V6 hypervariable 16S rRNA gene regions was conducted. The variable regions were amplified using primers 807F and 1050R [403], purified, and re-amplified to add barcodes and adapters for Illumina sequencing as previously described [404]. Libraries were sequenced using 250 bp paired-end sequencing chemistry on an Illumina

MiSeq platform. The average quality was assessed by sliding a window of 10% of the read length along the sequence. Read fragments that had a PHRED score of the fastq file (Q value) below 10 were removed. Reads shorter than 140 nt after trimming were removed as well. Also reads that had an N character in their sequence, any mismatches within the primers and barcodes or more than 10 homopolymer stretches were discarded. Then reads were trimmed conservatively to 140 nt and the paired ends subsequently matched to give 280 nt. Reads were first collapsed and clustered together allowing for 2 mismatches using Mothur [405]. A representative read was further considered if (i) it was present in at least one sample at a relative abundance of >1% of the total sequences of that sample or (ii) it was present in at least 2% of samples at a relative abundance of >0.1% or (iii) it was present in at least 5% of samples. This way, a total of 473 phylotypes or operational taxonomic units (OTUs) were resolved for further analysis. The paired-end reads (140 nt) of each OTU were merged to fully cover the V5-V6 region. One mismatch was allowed in the overlapping of the forward and reverse reads and the length of the retrieved sequences was ranging from 235 – 251 nt.

Phylogenetic analysis

Previously, the predominant faecal microbiota from cheetahs B1 and B2 was characterized by the phylogenetic analysis of clone libraries [351]. For comparative purposes, all OTUs obtained from Sanger sequencing in that previous study and those obtained from Illumina MiSeq in the present study were taxonomically assigned in a similar manner based on comparison against The Ribosomal Database Project-II (RDP) database using the RDP Naïve Bayesian Classifier [406]. Only annotations with a bootstrap value over 0.8 were considered as well identified phylogenetic levels, leaving following levels as undefined. In addition to RDP annotations, closest type strains were assigned to each of the 473 OTUs obtained with Illumina MiSeq using the EzTaxon server (http://www.ezbiocloud.net/eztaxon; [355]). For all samples, alpha (within-sample) diversity was estimated by calculation of the Shannon Diversity Index and analysis of rarefaction curves using QIIME [219]. The MiSeq rarefaction curves appeared to reach saturation (**FIG S6.1**), indicating that sequencing provided us with a nearly complete inventory of the bacterial 16S rRNA gene sequences present in the samples B1_2 and B2_2. Animal B2 exhibited the highest species richness as evident from the respective curve reaching a higher plateau and the higher Shannon index for B2. A similar result was obtained by rarefaction analysis of the data set previously obtained by 16S rRNA gene clone libraries [351]. Data statistics of both methods have been summarized in **Table S6.1.**

Data analyses and visualisation

To remove sampling depth heterogeneity, OTU tables generated for both samples B1_2 and B2_2 and OTU tables generated for the ensemble of time series samples were rarefied to the number of reads present in the sample with the lowest amount of reads (*i.e.* 53 753 and 46 083 reads per sample, respectively). Pareto-Lorenz (PaL) evenness curves were also constructed, based on the OTU profiles of previously generated clone libraries and Illumina generated data. For each data set, OTUs were ranked

from high to low based on their relative abundance. The cumulative proportion of OTUs and their respective cumulative abundances were used to graphically represent the structure of the bacterial community. The curves were numerically interpreted by the GINI coefficient (a single value between 0 and 1, measuring the normalized area between a given Lorenz curve and the perfect evenness line) [407] and the functional organization index (*Fo*), given by the horizontal y-axis projection on the intercept with the vertical 20% x-axis line [356]. The more the PaL curve deviates from the 45°C diagonal line (*i.e.* the theoretically perfect evenness line), the higher the GINI coefficient and the more uneven a community is [407,408].

Beta (between-sample) diversity was estimated for time series samples by calculation of both weighted and unweighted UniFrac distances [409] and further visualized by principal coordinates plots (PCoA) in QIIME. To explore taxonomic driving factors of patterns in PCoA plots, biplots were produced where bacterial taxa are plotted in the same PCoA space and spheres represent the relative abundances of the taxon in the samples. The variation in community structure obtained by Illumina sequencing was further compared to principal component analysis (PCA) performed on the fingerprint data set of these time series samples with the score plot illustrating *how* the samples are related and the loading plot *why* the samples are related.

To permit summarized comparisons across the data resulting from clone libraries and Illumina sequencing of two samples from cheetahs B1 and B2, relative abundances at different phylogenetic levels were calculated. *Ad hoc* definitions were used to classify OTUs as abundant or rare in relation to their relative abundances. Abundant OTUs were defined with relative abundances ≥1%, whereas rare OTUs were defined with abundances <1% [410,411]. To assess temporal patterns in microbial diversity, relative proportions of rare and abundant OTUs were plotted over the observation period. Furthermore, microbial community dynamics over this two-year period was visualized for each animal as the proportion of shared abundant and rare OTUs between consecutive sample collections. For the abundant OTUs, a heatmap was also created in R [412] showing their relative abundances across all time series samples from PL and OV. To determine if any groups of samples (based on provenance) contained significantly different bacterial communities, analysis of similarity (ANOSIM) was used on the unweighted UniFrac distance matrix. Differences in the proportions of OTUs (defined as percentage of total reads) between PL and OV were determined using non-parametric Kruskal-Wallis tests ($\alpha = 0.05$), and all 473 OTUs were included in analysis.

6.3. Results and Discussion

Using different subsets of the total data set generated by Illumina MiSeq sequencing, three types of comparative analyses were performed. First, Illumina data were compared to those obtained with phylogenetic clone libraries [351] using the same samples (*i.e.* B1_2 and B2_2) for both methods. Secondly, within-animal variation over time was addressed using NGS sequencing data from time series samples collected in five cheetahs that had been previously analyzed by integrated community profiling in combination with a series of real-time PCR assays. Finally, variable phylogenetic diversity across both zoo environments assessed by cross-sectional analyses of community profiling [401] were compared to NGS sequencing data.

6.3.1. Cheetah faecal microbial diversity: comparing Illumina MiSeq sequencing with cloning

The single samples from the two cheetahs housed at PL comprised 53 754 sequence reads for sample B1_2 and 56 501 for sample B2_2, totalling 110 255 usable paired-end sequence reads with on average 246 nt read length. Reads clustered into 156 OTUs. Although using different amplification and sequencing primers, taxonomic information at phylum and order level obtained through cloning was qualitatively similar to results obtained from Illumina sequencing across both samples. Although the V3 region which was used as target of previous clone library analyses [351] performs better than the V5-V6 region in the taxonomic attribution with the RDP Classifier at stringent bootstrap cut-off values [413], only 4.7% of Illumina reads remained unclassified at the family level. However, taxonomic classification of 99% sequence identity clusters often shows a distinct taxonomic shift when comparing predominant to low-abundance clusters. The increase in unclassified sequences is larger for low-abundance clusters within a taxonomic level and increases with depth of classification [414]. Thus, the 4.7% of unclassified reads may harbor members of the rare biosphere with potential roles in ecosystem homeostasis [415]. It must also be noted that, due to the stringency of data preprocessing, part of the low-abundant phylotypes were already removed, which might explain why only from family level on unclassified sequences were detected.

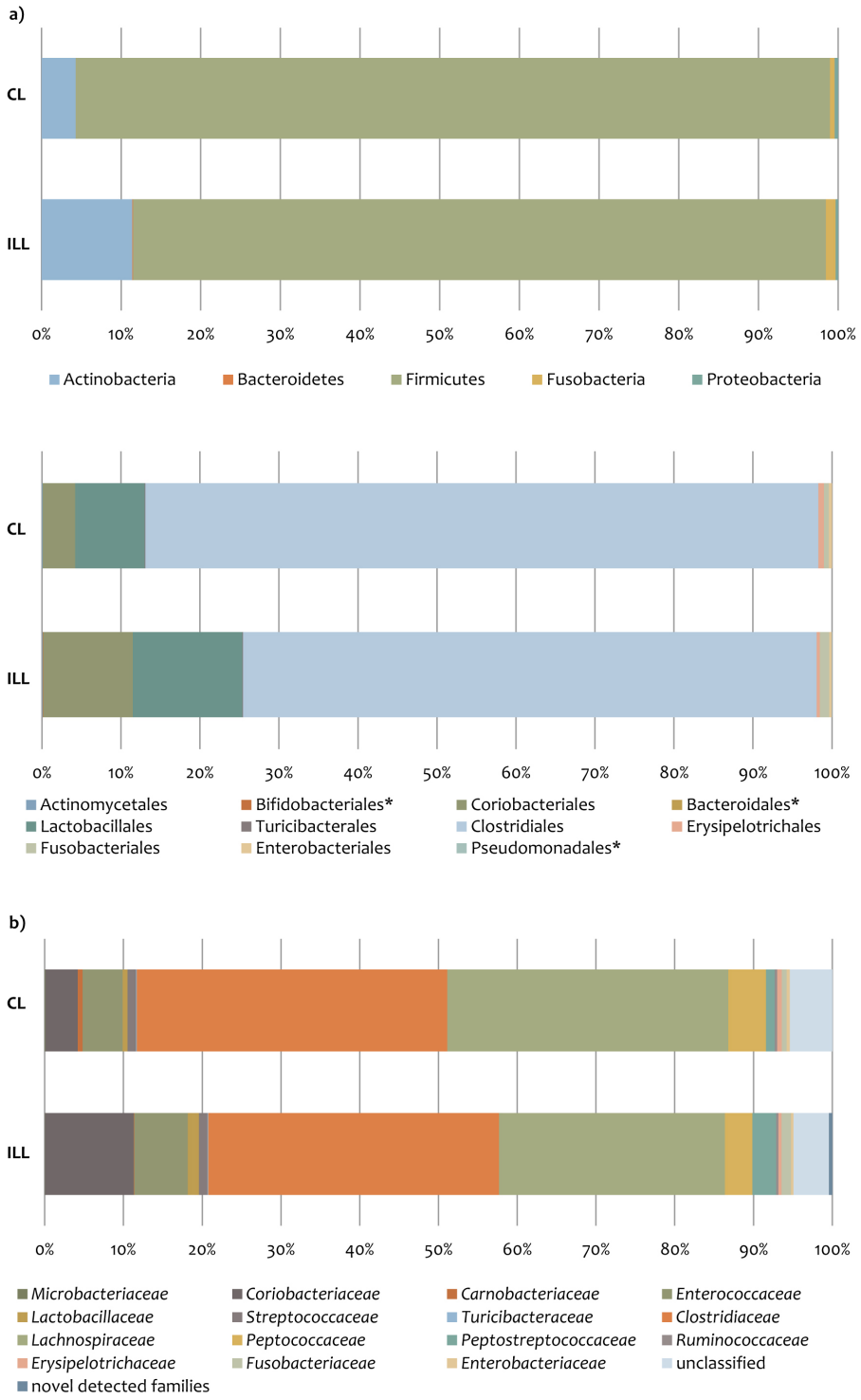


FIG 6.1 | Phylogenetic diversity at a) phylum and order level and at b) family level as revealed by 16S rRNA Sanger clone libraries and Illumina sequencing of two faecal samples from two captive cheetahs. Three orders detected additionally by Illumina sequencing are marked with an asterisk. The novel detected families include *Actinomycetaceae, Corynebacteriaceae, Nocariaceae, Bifidobacteriaceae, Bacteroidaceae, Leuconostocaceae, Veillonellaceae, Moraxellaceae and Pseudomonadaceae*.

The majority of the reads belonged to the phylum Firmicutes and to the orders Clostridiales and Lactobacillales (**FIG 6.1a**). The same groups were previously also shown to constitute the majority of faecal cheetah-associated phylotypes by clone library analysis and Sanger sequencing [86,351] and by oligotyping of Illumina MiSeq generated reads [264]. In contrast to clone library analysis, amplicon sequencing detected members of the phylum Bacteroidetes, albeit with a very marginal share (*i.e.* 0.1%) of the reads. In addition, Illumina MiSeq also revealed a larger share of Actinobacteria (*i.e.* 7.2% extra compared to their abundance in clone libraries). The latter finding is not surprising since it is well known that high-GC taxa such as Actinobacteria are often underrepresented in 16S rRNA gene clone libraries [342,416]. At the ordinal level, 3 additional orders not previously found by clone library analysis were detected additionally by NGS sequencing, but together covered only 0.1% of the reads (**FIG 6.1a**).

At family level, 9 families which covered together encompassed 0.43% of the reads were additionally detected. The relative abundances of the previously detected families varied greatly between both methods (**FIG 6.1b**). Reads unclassified at family level clustered into 20 OTUs. Taxa that were previously not discovered in these samples by clone library analysis represented only 11 OTUs, *i.e.* 0.3% of the total read number of the Illumina-generated data set. Collectively, these observations seem to indicate that the majority of the taxonomic diversity from phylum to family level first revealed by clone library analysis corresponds to the new findings from Illumina MiSeq. Beyond these levels, however, the NGS approach achieved a greater depth of coverage and revealed higher phylotype richness. Although many taxa were identified in the two samples included in this first analysis, only a few dominated. In fact, 2 phyla (Firmicutes and Actinobacteria), 3 orders (Coriobacteriales, Lactobacillales and Clostridiales) and 4 families (*Coriobacteriaceae*, *Enterococcaceae*, *Clostridiaceae* and *Lachnospiraceae*) contributed to more than 80% of the reads at the respective taxonomic levels. Conversely, many rare taxa contributed only a few sequences each. For both clone library and Illumina approaches, the functional organisation (*Fo*) of bacterial communities as inferred from Pareto-Lorenz curves of cumulative OTU abundances supported this specialized overall community structure in which a small amount of the phylotypes is dominant and all others are present in low numbers. Upon taxonomic binning of both samples 20% of the OTUs recovered by clone library analysis encompassed 81% of the cumulative sequence abundances and this number further increased to 94% for the Illumina-generated data (**FIG 6.2**). Likewise, high GINI coefficients of 0.73 for clone libraries and 0.88 for Illumina-generated data underpin the uneven distribution in the community.

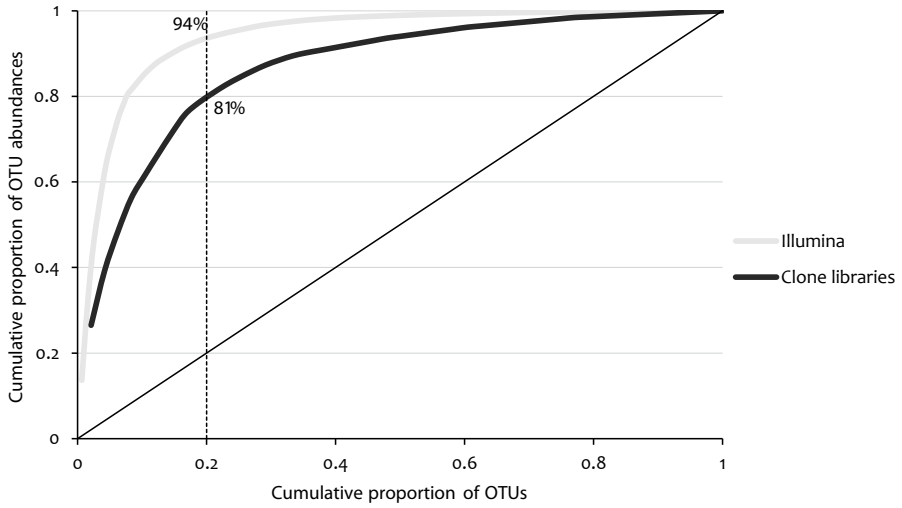


FIG 6.2 | Pareto-Lorenz curves derived from 16S rRNA phylogenetic clone library analysis [351] **and Illumina sequencing of faecal samples from two captive cheetahs.** The 45° diagonal represents the perfect evenness of a community. The dashed vertical line at the 0.2 x-axis level is plotted to evaluate the *Fo* index.

6.3.2. Cheetah faecal microbial temporal stability: comparing Illumina MiSeq sequencing with integrated DGGE profiling combined with real-time PCR assays

All 25 time series samples from two cheetahs housed at PL (B1 and B2) and three cheetahs housed at OV (NL9, NL10 and NL11) comprised a minimum of 46 083 sequence reads. The mean number of sequences per sample was 62 038 ± 8244, totalling 1 550 961 paired-end sequence reads which clustered into 473 OTUs. Data statistics and rarefaction curves for all animals are summarized in **TABLE S6.2** and **FIG S6.2**. Rarefaction curves depict differences in faecal microbiota richness between animals, but also indicate a high coverage for each sample with saturation levels at ~20 000 reads.

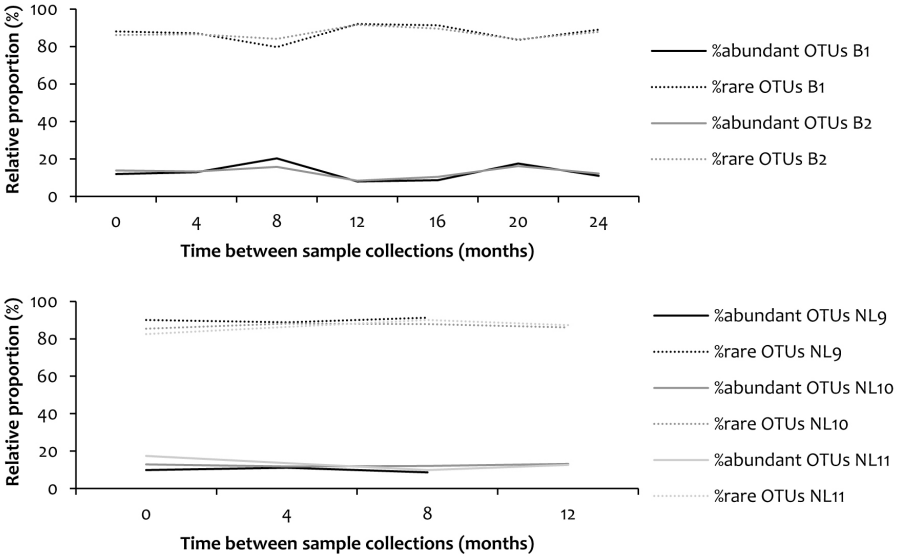


FIG 6.3 | The proportion of abundant (≥1%) and rare (<1%) OTUs derived from Illumina data depicted at four-monthly sampling intervals. Cheetahs B1 and B2 were housed at Zoo Parc Planckendael (PL, Belgium) and cheetahs NL9, NL10 and NL11 at Zoo Parc Overloon (OV, the Netherlands).

Over a time period of 12 to 24 months, the proportions of abundant (≥1% of reads) and rare OTUs (<1% of reads) were relatively constant across the five cheetahs under study (**FIG 6.3**). However, on average only 58% of OTUs delineated from Illumina data was shared between consecutive samples over all four-monthly sampling intervals. One exception to this is animal NL9 for which a greater shift in community structure was observed, with on average only 33% of OTUs shared between samples (**FIG 6.4a**). When excluding abundant OTUs from the data set, percentage of shared OTUs between consecutive sampling points decreased to an average of 43% and clearly showed the higher temporal variability in low abundant OTUs (**FIG 6.4c**). In contrast, the percentage of shared abundant OTUs between consecutive sampling dates was on average 85% (**FIG 6.4b**). Only a small number of OTUs (*i.e.* OTU1, OTU2 and OTU4) were stably detected in all samples over a two-year period. Upon comparison with the type strain entries in EzTaxon, these were most closely affiliated with *Ruminococcus gnavus* ATCC 29149^T(98.78%), *Clostridium hiranonis* TO-931^T (99.59%) and *Blautia glucerasea* HFTH-1^T (99.18%), respectively. Moreover, the high proportion of the total reads (*i.e.* 41%) belonging to these three OTUs revealed by depicting their abundances per animal in a heatmap (**FIG 6.5**) indicates that they are members of the phylogenetic core of the faecal microbiota in the captive cheetahs under study.

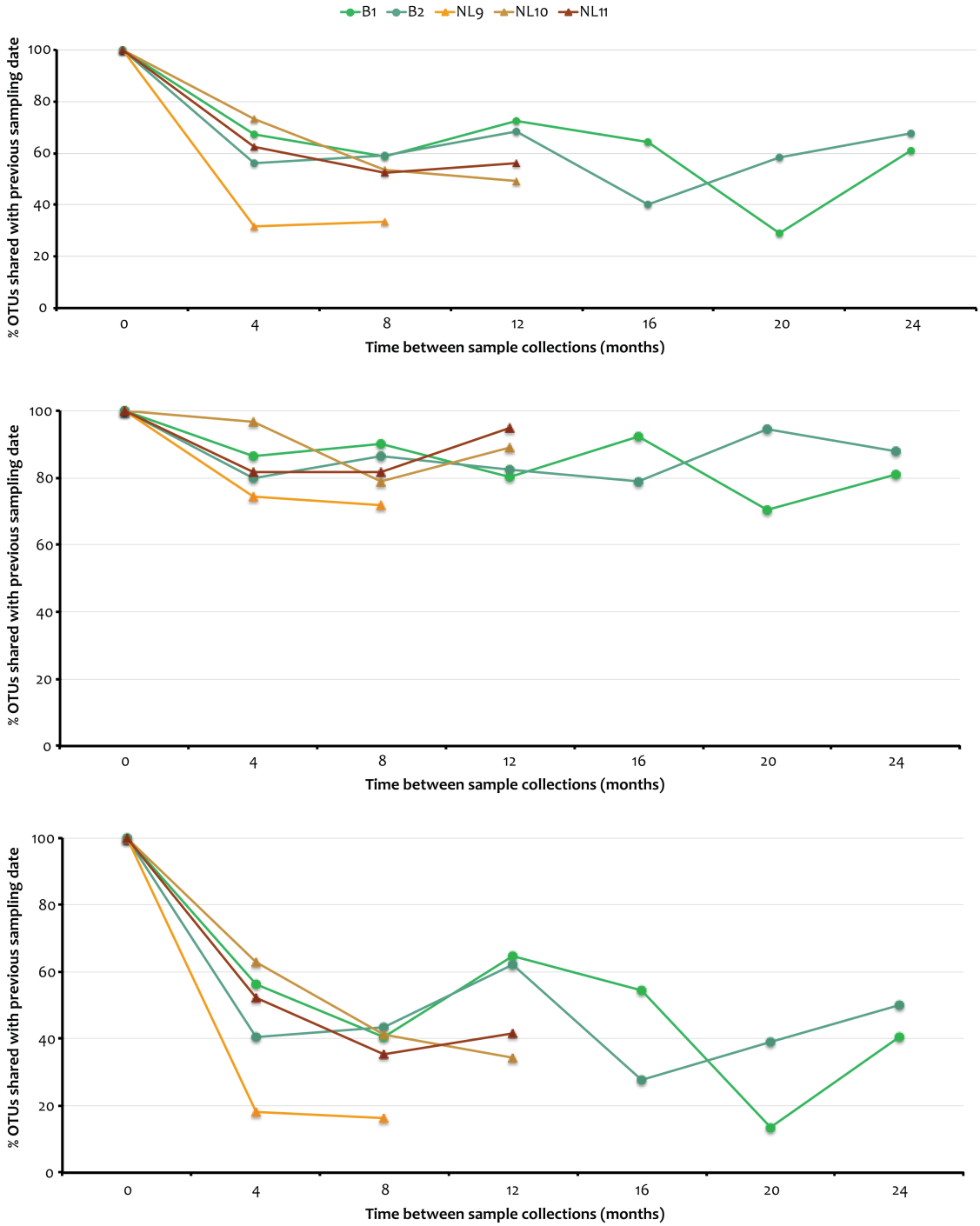


FIG 6.4 | Temporal dynamics of OTUs derived from Illumina data between consecutive sample collection dates. The percentage of shared OTUs was calculated between two successive sampling dates and was compared between animals considering (A) all OTUs, (B) abundant OTUs (≥1% of reads in min. 1 sample) and (C) rare OTUs (<1% of reads). The first sampling date is indicated as 100% of shared OTUs with themselves.

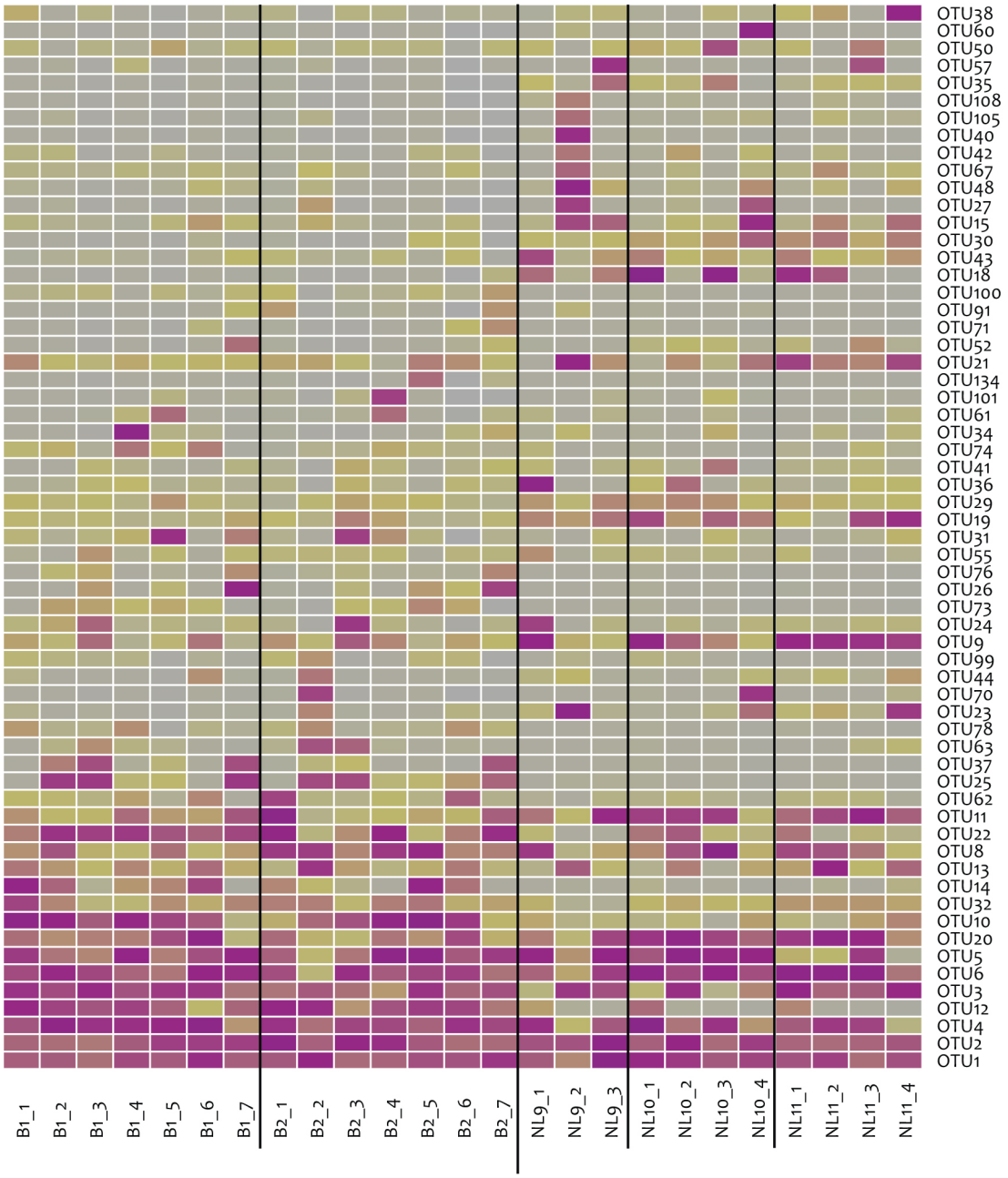


FIG 6.5 | Heatmap showing the relative abundance of OTUs derived from Illumina data present at ≥1% of reads in at least one of the faecal samples. Samples were collected at four-monthly intervals. Cheetahs B1 and B2 were housed at Zoo Parc Planckendael (PL, Belgium) and cheetahs NL9, NL10 and NL11 Zoo Parc Overloon (OV, the Netherlands). The heatmap represents the relative percentage of each OTU within each sample.

Also in case of the time series samples, Illumina sequencing provided an overall deeper coverage as evidenced by the different temporal dynamics between rare and abundant OTUs. On the other hand, our Illumina data appear to reinforce previous conclusions on the temporal stability of abundant OTUs obtained with an integrated DGGE approach. For the same samples, community profiling disclosed a stable faecal microbiota with 45.45% of the band-classes shared between all samples and a 23.3-25.6% change between consecutive samples [401]. As shown in the present study, these patterns are highly consistent with the temporal distribution of abundant OTUs recovered by Illumina sequencing.

Compared to the other cheetahs, consecutive samples of NL9 showed a remarkably lower portion of OTUs shared between consecutive sampling points and thus a higher variability in both rare and abundant OTUs (**FIG 6.6**). Also, total richness in these samples was reduced as shown by a decrease in Shannon's index and number of bands in the previously determined DGGE profiles [401]. The fact that the relative proportion of rare and abundant OTUs has not changed is a significant indication that variations not only occur due to loss of phylogenetic groups, but also due to reorganized relative abundances of OTUs already present. Real-time PCR assays previously showed changes in two key members of the cheetah faecal microbiota in samples of NL9, *i.e.* an increase (+0.9 log10 CFU/g) in *Clostridium* cluster I members and a decrease (-1.9 log10 CFU/G) in *Clostridium* cluster XIVa members [401]. These shifts were corroborated by the heatmap interpretation of the respective samples of NL9 (*i.e.* NL9_1 and NL9_2) (**FIG 6.5**). Here, a decrease in abundances of OTUs taxonomically assigned to *Clostridium* cluster XIVa (*i.e.* OTU1, OTU4, OTU6 and OTU20) coincides with an increase of OTUs assigned to *Clostridium* cluster I (i.e. OTU15, OTU48, OTU67). The case of NL9 illustrates that specific ecosystem perturbations detected by a conventional DGGE approach at a high taxonomic level cohere with findings revealed by contemporary sequencing methodologies.

Similar cases of marked correspondence between first-generation molecular tools and NGS have been reported. For instance, a combination of fingerprinting techniques, quantitative PCR and 454 Pyrotag sequencing has revealed a highly comparable overall community structure in sediment samples [417]. DGGE-based predominant lineage identifications were in agreement with those found using 454 pyrosequencing in tap water samples [418], and marked similarities at the level of bacterial orders were observed between cloning and 454 pyrosequencing of gut microbial communities in ants [396]. As recently reviewed by Lynch and co-workers [415], the true added value of next-generation sequencing lies in its ability to more finely disentangle data sets and yield insights into unkown microbial taxa and the rare biosphere. From observations in a wide range of ecosystems (e.g. air; marine; adult human skin, tongue and gut), it is already clear that NGS can provide a framework for improved observational investigations of ecosystem dynamics [419]. It is likely that exploring the dynamics of rare taxa can play an important role in our understanding of subtle changes in temporal variability and the functional perturbations of gut microbial communities upon e.g. dietary supplementation, medication, gastrointestinal and metabolic diseases. For instance, from metadata collected during sampling of NL9, we know that this cheetah temporarily suffered from gastrointestinal disease, including diarrhoea and reduced appetite. The changes in microbial key groups and low-abundance OTUs observed using integrated DGGE and Illumina MiSeq might therefore reflect a state of dysbiosis resulting from compromised health or temporary disturbance upon administration of antibiotics or a combination thereof.

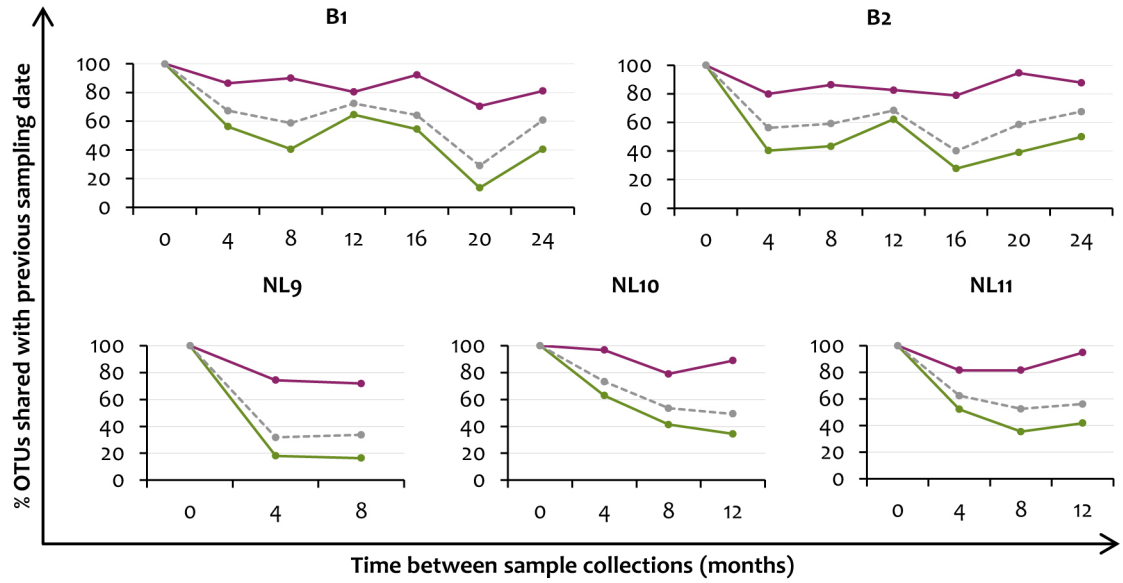


FIG 6.6 | Temporal dynamics of the faecal microbiota of 5 captive cheetahs based on Illumina data. The dashed line represents the portion of OTUs shared between consecutive sampling points considering all OTUs, whereas the purple line illustrates the shared portion of abundant OTUs (≥1% of reads) and the green line the shared portion of rare OTUs (<1% of reads).

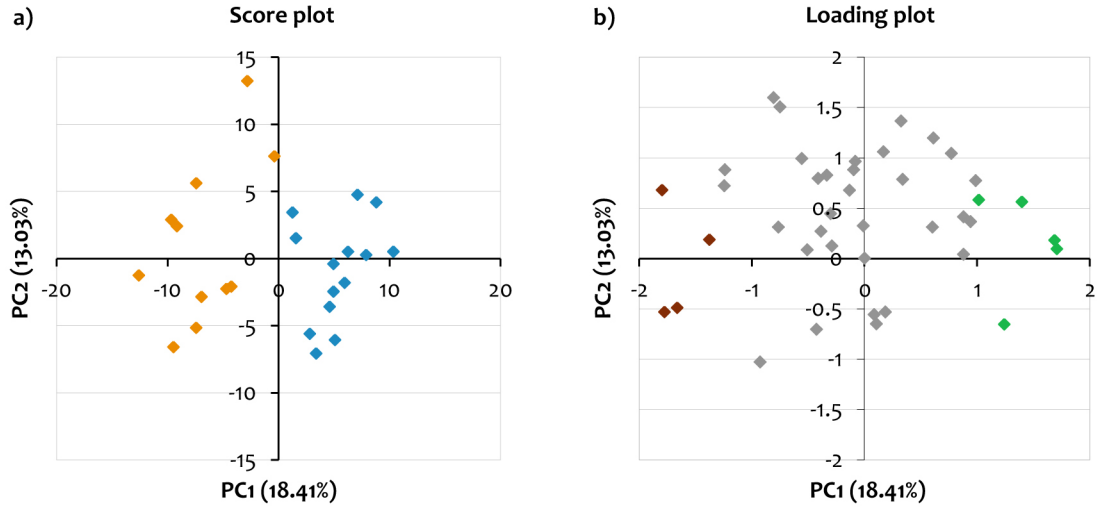


FIG 6.7 | PCA plots of DGGE fingerprints[a] derived from faecal samples of 3 cheetahs housed at Zoo Parc OV and 2 cheetahs housed at Zoo Parc PL. A) Score plot depicting variance between samples from cheetahs in OV (yellow) and PL (blue). B) Loading plot depicting variables, defined as band-classes (grey), driving the observed pattern between samples. Red dots include band-classes found at higher intensities in fingerprint profiles derived from OV (taxonomically assigned to *Erysipelotrichaceae*, *Lactobacillaceae*, *Clostridium* cluster XIVa and *Clostridium* cluster I)[a]. Green dots include band-classes found at higher intensities in fingerprint profiles derived from PL samples taxonomically assigned to *Streptococcaceae*, *Enterococcaceae*, *Clostridium* cluster I, XI and XIVa).
[a] Data from [351]

6.3.3. Comparison of zoo sample sets using conventional molecular tools and Illumina MiSeq sequencing

In our previous study, community profiling indicated no significant differences at animal level, but displayed a number of differences between PL and OV sample sets [401]. PCA of molecular fingerprints of the samples included in the present study visualized the separate clustering of PL and OV samples (**FIG 6.7a**), mainly driven by banding patterns which have been found significantly different in proportional composition between PL and OV sample sets (**FIG 6.7b**) [401]. When community variation in the Illumina data set was visualized by PCoA plots using unweighted UniFrac distances, similar microbial community structure patterns and zoo sample set-specific clustering were obtained (**FIG 6.8**). Analysis of similarity revealed that OTU composition and abundance was significantly different between both zoo data sets (ANOSIM, p=0.001). Taking into account all 473 OTUs, a selection of 25 OTUs were significantly different between data sets of PL and OV and explained this clustering of samples according to provenance **TABLE S6.3**.

Importantly, this OTU selection encompassed a number of discriminating phylogenetic groups as previously revealed by discriminant analysis of DGGE fingerprints [401]. From these 25 OTUs, only 7 covered more than 1% of the reads each in at least one of the populations. Again, this illustrates the potential of Illumina MiSeq to detect low-abundance OTUs that contribute to the separation of host-associated communities exposed to different environmental conditions.

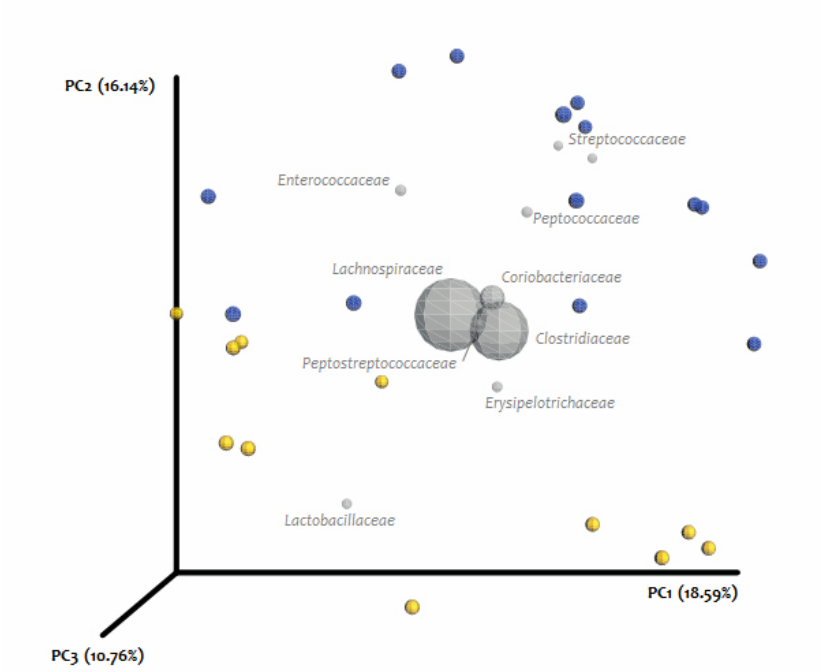


FIG 6.8 | PCoA plot of unweighted UniFrac distances derived from Illumina data. Samples from OV (yellow) cluster away from samples from PL (blue). Superimposed are grey spheres indicating the most abundant bacterial families. The sizes of the spheres represent the mean relative abundance of the respective taxon and the location of the spheres within the plot indicate subject-specific associations.

The central position of the most common families in the PCoA plot, *i.e. Lachnospiraceae*, *Clostridiaceae* and *Coriobacteriaceae* (**FIG 6.8**), indicates that these are likely candidate members of the faecal core microbiota of captive cheetahs. The first two families include *Clostridium* clusters I, XI and XIVa which have been previously detected by real-time PCR assays at 7-8 log10 CFU/g and are present in >80% of all sample profiles generated by DGGE [401]. These shared highly abundant families were represented by 9 OTUs, which in EzTaxon appeared most closely to *Ruminococcus gnavus* ATCC 29149^T, *Clostridium hiranonis* TO-931^T, *Blautia schinkii* DSM 10518^T, *Collinsella aerofaciens* JCM 10188^T and *Collinsella intestinalis* DSM 13280^T. Accordingly, *Coriobacteriaceae*, *Clostridium* clusters XIVa and XI make up a substantial part of the faecal microbiota in domestic cats [272].

6.4. Conclusions

The evaluation of new Illumina MiSeq data against results previously obtained with conventional molecular tools primarily aimed to obtain a multivariate view on the phylogenetic diversity and temporal stability of the faecal microbiota of captive cheetahs. Whereas the existing clone library served as a valuable reference of the core microbiome members of the cheetah gut, Illumina-based analyses of the same samples refined the proportional abundances of the detected phyla and their phylotype richness, and also revealed a marginal share of previously undetected diversity. On the other hand, the combined 16S rRNA DGGE-clone library method and the more in-depth Illumina MiSeq data set yielded a similar shortlist of variables explaining faecal community changes across different animals housed at different environments. Also here, the latter approach provided a more enhanced view on microbial dynamics by revealing subtle temporal variations in the rare or less abundant taxa.

Whereas there was a remarkable coherence between conventional molecular ecology tools such as phylogenetic clone libraries and DGGE and a contemporary sequencing approach like Illumina MiSeq to inventorize and monitor major lineages in the cheetah's gut microbiota, our study has illustrated that the genuine added value of NGS seems to be the discrimination between closely related communities of conspecific hosts. However, deeper sequencing does not necessarily come with deeper understanding of the gut microbial diversity and dynamics. To go beyond cataloguing, to validate the biological meaning of observed patterns and to further examine the functional role of the cheetah gut microbiome, an integration of traditional molecular techniques for rapid and low-cost community screening with NGS for in-depth analysis as employed in the current study is only a starting point [222]. To further address these questions, the toolbox of the microbial ecologist will also require (un)targeted metabolic profiling and well-designed and -controlled *in vitro* models for ecologically significant interpretation of phylogenetic information.

SUPPLEMENTARY DATA

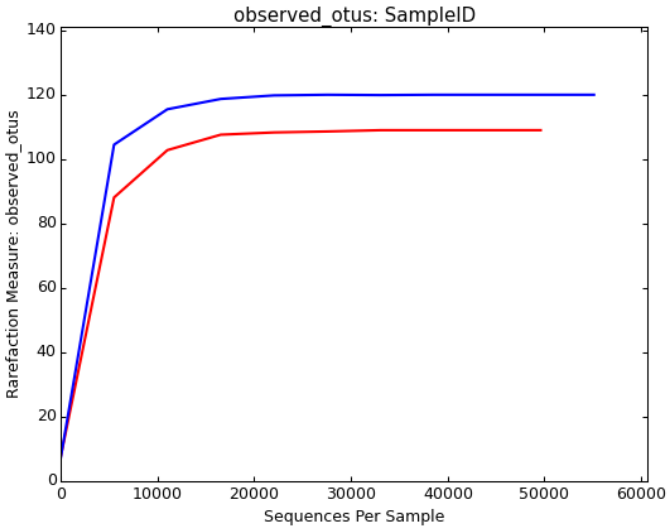


FIG S6.1 | Rarefaction curves for bacterial V5-V6 16S rRNA gene sequences obtained by Illumina MiSeq sequencing of two captive cheetah faecal samples. B1 (red), B2 (blue)

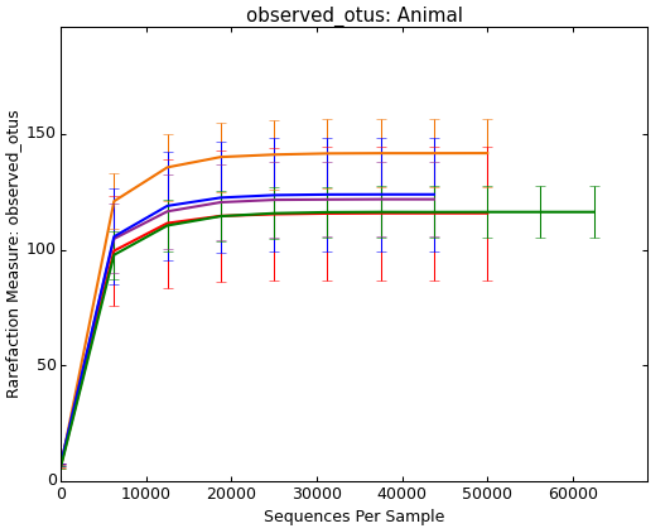


FIG S6.2 | Rarefaction curves for bacterial V5-V6 16S rRNA gene sequences obtained by Illumina MiSeq sequencing of captive cheetah time series faecal samples. B1 (red), B2 (blue), NL9 (orange), NL10 (green), NL11 (purple)

TABLE S6.1 | Statistics of 16S rRNA Sanger clone libraries and Illumina MiSeq data generated from two faecal samples (i.e. B1_2 and B2_2) from cheetahs B1 and B2, respectively. [a] derived from [351]

16S rRNA gene Clone libraries[a]			Illumina MiSeq (V5-V6 16S rRNA)		
	B1_2	**B2_2**	**B1_2**	**B2_2**	
total number of quality checked clone sequences	352	350	53754	56501	total number of clean reads
sequence length	400-450 bp	400-450 bp	246 bp	246 bp	sequence length
shared OTUs	18	18	73	73	shared OTUs
animal-specific OTUs	11	19	36	47	animal-specific OTUs
total OTUs	29	37	109	120	total OTUs
Shannon Diversity Index	3.77	3.44	4.12	4.32	Shannon Diversity Index

TABLE S6.2 | Statistics of Illumina MiSeq data generated for a series of faecal samples from cheetahs B1 and B2, housed at Zoo Parc Planckendael (PL, Belgium) and cheetahs NL9, NL10 and NL11, housed at Zoo Parc Overloon (OV, the Netherlands).

Zoo	Animal	Sample ID	Shannon Diversity Index	# clean reads	# OTUs (99%)	# abundant OTUs[a]	# rare OTUs[b]	% abundant OTUs	% rare OTUs
PL	**B1**	B1_1	4.03	57432	101	12	89	11.88	88.12
		B1_2	4.12	53754	109	14	95	12.84	87.16
		B1_3	4.55	56669	94	19	75	20.21	79.79
		B1_4	4.03	60061	151	12	139	7.95	92.05
		B1_5	3.89	69236	162	14	148	8.64	91.36
		B1_6	3.61	56671	74	13	61	17.57	82.43
		B1_7	3.87	78889	119	13	106	10.92	89.08
	B2	B2_1	3.62	46083	87	12	75	13.79	86.21
		B2_2	4.32	56501	120	16	104	13.33	86.67
		B2_3	4.84	58320	152	24	128	15.79	84.21
		B2_4	4.07	56232	157	13	144	8.28	91.72
		B2_5	3.86	62488	106	11	95	10.38	89.62
		B2_6	4.13	65706	105	17	88	16.19	83.81
		B2_7	4.42	76822	140	17	123	12.14	87.86
OV	**NL9**	NL9_1	4.26	68339	161	16	145	9.94	90.06
		NL9_2	3.99	71980	125	14	111	11.20	88.80
		NL9_3	3.29	55608	139	12	127	8.63	91.37
	NL10	NL10_1	3.33	64900	101	13	88	12.87	87.13
		NL10_2	3.56	64981	118	14	104	11.86	88.14
		NL10_3	4.28	64304	132	16	116	12.12	87.88
		NL10_4	3.84	62974	114	15	99	13.16	86.84
	NL11	NL11_1	4.14	46330	109	19	90	17.43	82.57
		NL11_2	3.98	75150	103	14	89	13.59	86.41
		NL11_3	4.29	64124	141	14	127	9.93	90.07
		NL11_4	4.19	57407	134	17	117	12.69	87.31

[a] abundant OTUs defined at ≥ 1% of reads

[b] rare OTUs defined at 0-1% of reads

TABLE S6.3 | Significantly different OTUs between faecal samples from cheetahs housed in Zoo Parc Planckendael (PL) and cheetahs housed in Zoo Parc Overloon (OV), assessed by Illumina sequencing and compared with discriminating band-classes from DGGE profiles.[b] derived from [401]

OTU	*P* -adjusted[a]	Relative abundance in PL (% of reads)	Relative abundance in OV (% of reads)	Closest type strain (% similarity)	Discriminating band-classes from DGGE profiles[b]
OTU35	0.0036	0.000	0.444	*Enterorhabdus mucosicola* Mt1B8^T (97.08%)	
OTU45	0.0036	0.000	0.297	*Allobaculum stercoricanis* DSM 13633^T	x (Bcl 39.81)
OTU30	0.0046	0.052	0.941	*Coprobacillus cateniformis* JCM 10604^T	x (Bcl 39.81)
OTU12	0.0046	4.412	0.319	*Clostridium hiranonis* TO-931^T	
OTU78	0.0046	0.280	0.001	*Peptostreptococcus stomatis* W2278^T	x (Bcl 78.65)
OTU100	0.0077	0.216	0.000	*Dorea longicatena* AJ132842^T	
OTU43	0.0101	0.118	0.877	*Blautia glucerasea* HFTH-1^T	
OTU198	0.0101	0.024	0.001	*Cryptobacterium curtum* ATCC 800683^T	
OTU520	0.0101	0.000	0.022	*Ruminococcus gnavus* ATCC 29149^T	
OTU104	0.0101	0.000	0.135	*Collinsella tanakaei* YIT 12063^T	
OTU18	0.0114	0.013	2.103	*Coprococcus comes* ATCC 27758^T	
OTU187	0.0201	0.152	0.017	*Clostridium hiranonis* TO-931^T	x (Bcl 80.93)
OTU467	0.0213	0.038	0.003	*Clostridium hiranonis* TO-931^T	x (Bcl 80.93)
OTU236	0.0213	0.019	0.000	*Collinsella aerofaciens* JCM 10188^T	
OTU76	0.0213	0.325	0.000	*Leuconostoc mesenteroides* NCFB 529^T	
OTU146	0.0216	0.001	0.037	*Arcanobacterium hippocoleae* CCUG 44697^T	
OTU22	0.0221	3.352	0.538	*Peptococcus niger* X55797^T	
OTU10	0.0221	4.985	0.439	*Collinsella aerofaciens* JCM 10188^T	
OTU503	0.0287	0.017	0.001	*Blautia schinkii* DSM 10518^T	
OTU14	0.0299	3.011	0.016	*Enterococcus cecorum* ATCC 43198^T	
OTU25	0.0305	2.096	0.004	*Lactobacillus sakei* DSM 20017^T	x (Bcl 38.50)
OTU37	0.0332	1.115	0.000	*Lactococcus plantarum* DSM 20686^T	x (Bcl 32.59)
OTU73	0.0332	0.396	0.000	*Holdemania filiformis* ATCC 51649^T	
OTU248	0.0407	0.000	0.014	*Acetobacterium carbinolicum* DSM 2925^T	x (Bcl 28.14)
OTU108	0.0407	0.000	0.133	*Holdemania filiformis* ATCC 51649^T	x (Bcl 39.81)

[a] *P*-values based on non-parametric Kruskal-Wallis tests ($\alpha = 0.05$)

7 FAECAL COMMUNITY PROFILING REVEALS CORE MEMBERS AND DIET-ASSOCIATED DIFFERENTIATION IN THE HOST MICROBIOTA OF CAPTIVE AND FREE-RANGING CONSPECIFICS OF THE CHEETAH (*Acinonyx jubatus*)

Mammalian gut microbiota compositions may differentiate according to host phylogeny, gut morphology and dietary habits. One of the strategies to explore the influence of these factors on host-microbe co-evolution and host-level differences is to investigate the natural variation in gut microbial community composition of captive wildlife species and their free-ranging conspecifics. Compared to omnivores and herbivores, the phylogenetic differentiation among microbiomes in carnivores has not been studied in great detail. In this study, we primarily focussed on the faecal microbiota of the largest population ever studied of the captive cheetah (*Acinonyx jubatus*), a strict carnivore with endangered status. For this purpose, phylogenetic profiling data obtained from 50 cheetahs housed at 13 European zoological institutions were mirrored to data obtained from a smaller subset of free-ranging Namibian conspecifics. Despite the obvious difference in environmental factors to which the two conspecific groups are exposed, community profiling with Illumina MiSeq sequencing, DGGE fingerprinting and quantitative PCR revealed similar microbial species richness and identified faecal core members belonging to *Clostridium* cluster XIVa (8.77-8.97 log10 CFU/g), *Clostridium* cluster I (8.12-8.13 log10 CFU/g) and *Clostridium* cluster XI. However, members of the latter cluster were represented in significantly greater number in captive cheetahs (7.28 log10 CFU/g) compared to free-ranging conspecifics (6.59 log10 CFU/g). Also, community profiles of captive cheetahs fed only whole chickens did not group with other conspecifics housed in zoos but clustered more closely to profiles of free-ranging cheetahs. This may indicate that changes in substrate availability depending on the supply of different prey components (e.g. viscera and feathers) may drive faecal community differentiation in captivity. Likewise, comparison with faecal samples collected in domestic cats also revealed remarkably different microbial structures between cats fed kibble diet and captive cheetahs fed raw meat diets. Remarkably, the faecal microbial composition of one cat feeding on raw meat more closely resembled that of captive cheetahs. These findings support the hypothesis that different dietary habits within members of the *Felidae* may differentiate feline microbiomes to a larger extent than previously thought.

7.1 Background

The microbial species assemblages that compose mammalian gut microbiota are deeply integrated into almost all aspects of mammalian health and outnumber host cells 10 to 1 [420]. Comparative metagenomic studies have revealed that gut microbial communities can vary across different scales, even within mamalian species which have been reported to harbor a stable core gut microbiota. Such variation can stem from heritable factors (e.g. host phylogeny, generation-to-generation transmission) and environmental factors (e.g. host diet and geography) [62,86,250,421]. In Ley and co-workers' pioneering study on the faecal microbiota of 59 terrestrial mammalian species living either in zoos or in their natural habitat, it was concluded that the gut microbial composition is largely influenced by host phylogeny, diet and gut morphology suggesting that these bacterial communities have codiversified with their respective hosts [86]. Muegge and co-workers suggested that the adaptation of the microbiota to diet is similar across different mammalian lineages and overall distribution of microbial species does not mirror mammalian phylogeny [250].

Compared to omnivores and herbivores, the phylogenetic differentiation among microbiomes in carnivores has not been studied in great detail. Except for a few strict carnivores such as bushdog, lion, cheetah, hyaena and polar bear [86], most of the currently available carnivorous microbiome data are derived from companion or laboratory cats [261,422]. Whereas the genomes of domestic cats have been compared to those of wildcats and other felines [4], their microbiomes have not been compared directly. However, the inherent variability present in natural systems suggests that investigating the gut microbiota of a broad range of conspecifics (*i.e.* members of the same phylogenetic species in the evolutionary tree of mammals) living in either their natural habitat or in captivity may provide further insight into the environmental resilience of the (co-)evolutionary partnership between the host and its microbiota. A small number of studies on birds, primates, Antarctic seals and grizzly bears have highlighted differences in the gut microbial richness and composition of hosts living in a captive environment compared with living in the wild [265,378,423–427]. Differential abundance of some specific bacterial taxa was correlated to different nutrient content (e.g. protein, fibre) between natural and commercial diets [265,424], although it was impossible to entirely untangle dietary factors from other environmental and host-health factors [265,378,425]. These findings of natural variation in symbiotic gut communities in conspecific hosts may indicate important host-level differences in bacterially mediated traits.

The cheetah (*Acinonyx jubatus*) is a strict carnivore with endangered status for which recent evidence has suggested that gut microbiota differences may exist between free-ranging and captive populations. Based on an oligotyping-based inventory targeting V4-16S rRNA gene regions of faecal bacterial communities in samples from free-ranging Namibian cheetahs [264], Fusobacteria (18.1%) and Bacteroidetes (5.8%) appeared to be enriched compared to data for captive cheetahs (1.2% and 0.1%, respectively) obtained in one of our previous studies [428]. In addition, comparison with literature data

from the domestic cat [261,272,285,422] indicates that different gut microbial constellations at phylum level may also exist between different *Felidae* host species.

In this cross-sectional study, we compared the faecal community composition across the largest population of adult captive cheetahs so far investigated in a single microbiota study (*i.e.* 50 adult cheetahs housed at 13 different zoos from 8 European countries), and mirrored these data against those obtained from a small population of free-ranging Namibian cheetahs. The study was conducted in two phases, both of which used data from household cats as a reference of a domesticated *Felidae* member. In a first approach, a subset of 15 single samples from captive cheetahs from 3 zoos were subjected to phylogenetic profiling with Illumina MiSeq sequencing to obtain a deeper insight in the faecal microbiota composition of the captive cheetah population. Secondly, the total sample set including 50 single samples from captive cheetahs and 8 from free-ranging Namibian conspecifics was subjected to DGGE community fingerprinting and a series of group-specific real-time PCR assays, a methodological framework previously used to assess the temporal stability of faecal microbiota in captive cheetahs over a 3-year period [401].

7.2 Materials & Methods

Ethics statement

This study was conducted non-invasively, without animal handling or any change in daily management and housing conditions of the animals. Fresh faecal samples were collected upon defaecation from adult cheetahs housed at 13 institutions that are all full members of the European Association of Zoos and Aquaria (EAZA, http://www.eaza.net/membership). Cheetahs were housed according to the Minimum Standards for the Accommodation and Care of Animals in Zoos and Aquaria and the EAZA Code of Practice. Prior permission for faecal sampling was obtained from the staff veterinarians and mammal curators.

For free-ranging cheetahs, faecal samples were collected opportunistically within 24h after defaecation from free-ranging Namibian cheetahs which are monitored in their home ranges by the Cheetah Conservation Fund (CCF, Namibia).

In addition, fresh faecal samples were collected upon defaecation from five domestic cats, randomly chosen from five households. Permission for faecal sampling was obtained from the owners.

Study population, sample collection and DNA isolation

Fresh faecal samples (100-200 g/animal) were collected immediately upon defaecation from 50 cheetahs housed at 13 European zoological institutions. These zoological institutions feed cheetahs either vitamin- and mineral supplemented chunks of meat or whole prey including fur, feather, skin, and internal organs (e.g. rabbit, chicken) or a combination thereof. This is in contrast to American zoological institutions, which traditionally include commercial (minced) meat diets for feeding (large) exotic felids. All cheetahs were routinely treated prophylactically for internal parasites. No medical or health

problems were reported or were apparent on remote examination during sampling. Animal records, medical history and dietary regime are summarized in **TABLE S7.1**. In addition, fresh faecal samples from 8 free-ranging Namibian cheetahs were included (area near Otijawango 21° 44' S, 18° 15' E). Genetic makeup, age and sex of these cheetahs were previously recorded, however, no additional information was available on health status of the animal or prey consumed at the time of sampling.

Five fresh faecal samples were also collected immediately upon defaecation from five domestic cats living in different households. Cats 1, 2, 4 and 5 were fed commercially-prepared kibble diets, whereas cat 3 received a home-prepared raw meat diet consisting of diverse parts of raw chickens (including feathers and bones), regularly supplemented with fresh chicken heart, liver and egg.

The protocol of Pitcher and co-workers [315] was used in a modified version [402] to extract total bacterial DNA from the faecal samples. DNA size and integrity were assessed on 1% agarose electrophoresis gels stained with ethidium bromide. DNA yields and purity were determined by measurements at 234, 260 and 280 nm using a Nanodrop 2000 spectrophotometer (Thermo, Scientific).

Illumina MiSeq sequencing

The faecal microbiota community of a subset of the 50 captive cheetahs included in this study was characterized with Illumina MiSeq sequencing targeting the V5-V6 hypervariable 16S rRNA gene regions. This subset included 15 samples from captive cheetahs, *i.e.* 2 from Zoo Parc Planckendael (PL, Belgium), 3 from Zoo Parc Overloon (OV, the Netherlands) and 10 from Reepark Ebeltoft Safari (EB, Denmark). Five samples from domestic cats were included as reference for domesticated felines. The variable regions were amplified using primers 807F and 1050R [403], purified, and re-amplified to add barcodes and adapters for Illumina sequencing as previously described [404]. Libraries were sequenced using 250 bp paired-end sequencing chemistry on an Illumina MiSeq platform and raw reads were filtered and analyzed as described in Chapter 6. A total of 515 phylotypes or operational taxonomic units (OTUs) were resolved for further analysis. *Ad hoc* definitions were used to classify OTUs as abundant or rare in relation to their relative abundances. Abundant OTUs were defined with relative abundances ≥1% in at least one sample, whereas rare OTUs were defined with abundances <1%, following other studies in bacteria [410,411].

Illumina MiSeq data analyses

Phylogenetic analysis

All OTUs obtained from Illumina MiSeq in the present study were taxonomically assigned based on comparison against The Ribosomal Database Project-II (RDP) database using the RDP Naïve Bayesian Classifier [406]. Only annotations with a threshold value over 0.8 were considered as well-identified phylogenetic levels, leaving following levels as unclassified. Closest type strains were assigned to OTUs using the EzTaxon server (http://www.ezbiocloud.net/eztaxon; [355]).

Assessment of alpha and beta diversity

Rarefaction curves were constructed from the estimated number of OTUs in each individual sample using observed species richness in QIIME [219]. Sequence libraries were rarefied to 40 519 (*i.e.* the size of the smallest sequence library included in the analysis) before calculating the Shannon index and observed species richness, enabling comparable estimates of alpha diversity. In addition, the GINI coefficient was calculated [407] for numerical interpretation of the evenness of the communities. Alpha metrics have been summarized in **TABLE S7.2** and rarefaction curves reaching plateau in **FIG S7.1**.

Beta (between-sample) diversity was estimated by calculation of unweighted UniFrac distances between all libraries based on the fraction of phylogenetic branch length shared by pairs of gut communities [409]. Unweighted UniFrac distances were then used for principal coordinates analyses (PCoA) in QIIME. To explore taxonomic driving factors of patterns in PCoA plots, biplots were created where bacterial taxa are plotted in the same PCoA space and spheres represent the relative abundances of the taxon in the samples. Samples and OTUs were also designated in a bipartite network to further illustrate how OTUs were partitioned across samples. A stochastic spring-embedded algorithm was used to cluster the OTUs and samples. Variation of gut communities (*i.e.* OTU composition) between the cheetah and cat population was assessed using ANOSIM statistics and non-parametric Kruskal-Wallis tests were used to identify OTUs with differing relative abundances between both host populations (α=0.05 and Benjamini-Hochberg correction for *P*-values). All 515 OTUs were included in analysis.

Community PCR and Denaturing Gradient Gel Electrophoresis (DGGE)

The variable V3 region of the 16S rRNA gene was amplified for subsequent molecular fingerprinting using the universal bacterial primers GC-F357 and R518 as previously described [401]. The resulting 16S rRNA gene amplicons were analyzed with DGGE fingerprinting (D-code System, Bio-Rad, Nazareth, Belgium) using a 35-70% denaturing gradient and further analyzed in BioNumerics software version 7.0 (Applied Maths, St-Martens Latem, Belgium) [401]. Band intensities were calculated from the peak area in the densitometric curves. All of the profiles were compared using the band-matching tool, and uncertain bands were included in the position tolerance settings. Bands were allocated to band-classes (Bcl) which have been defined from a collective analysis of 55 fingerprint profiles of captive cheetahs [401]. Taxonomic identity of Bcl of interest was inferred by position-based comparison with a selection of previously generated clones that together represent all phylotypes identified in two cheetahs with phylogenetic clone library analysis [351].

Real-time quantitative PCR (qPCR)

The four bacterial groups selected for specific 16S rRNA gene real-time PCR assays were based on the high abundance of *Clostridium* clusters I, XI and XIVa and near absence of *Bifidobacteriaceae* previously observed in a clone library analysis of the faecal microbiota of two captive cheetahs [351]. In addition, also the Firmicutes to Bacteroidetes ratio was determined. Real-time PCR primers and amplification conditions were performed as previously described [401].

qPCR and DGGE data analyses

Data obtained with real-time PCR assays were visualized in boxplots. Differences of bacterial log gene copy numbers for *Clostridium* clusters XIVa, XI and I between free-ranging, captive cheetah and domestic cat populations were analyzed using non-parametric Kruskal-Wallis test (α=0.05). Abundance of *Clostridium* clusters was also compared between cheetahs and domestic cats (Mannheim Whitney U test, α=0.05). Relative abundances of Firmicutes and Bacteroidetes were analyzed in a similar manner. An initial exploration of similarities between DGGE profiles of all samples (n=63) was performed by hierarchical cluster analysis with the unweighted pair group method with arithmetic mean (UPGMA) and using the Pearson correlation coefficient. Potentially discriminating Bcl between free-ranging and captive cheetah populations and between cheetahs and domestic cats were identified with non-parametric Mannheim Whitney U and Kruskal-Wallis tests (α=0.05). *P*-values were corrected for multiple-testing error by using the Benjamini-Hochberg method [357].

7.3 Results

7.3.1 Phylogenetic inventorization of the faecal microbiota across a subset of captive cheetahs and domestic cats with Illumina MiSeq

On average, 12.5% of the 515 OTUs detected in the total sample set (n=15 + 5) was present in less than 1% in each sample and another 21.5% of the OTUs was present in less than 5% in each sample (**FIG S7.2**). The relative abundance of the most abundant phylotypes (≥ 5% of the reads/sample) varied across individuals and across the captive cheetahs (n=15) and cats (n=5) which originated from three different zoos and five different households, respectively (**FIG 7.1**). In support of this observation, PCoA analysis based on unweighted UniFrac distances grouped samples based on host species (**FIG 7.2a**) whereas biplots showed the taxonomic families driving the observed clustering (**FIG7.2b**). ANOSIM analyses based on unweighted UniFrac distances underpin the significant differences in the faecal bacterial communities between the selected captive cheetahs and cats (R=0.90, *P*=0.001).

Additional Kruskal-Wallis tests identified 46 OTUs with significantly different abundance across host species, including 17 abundant OTUs (≥ 1% of reads) (*P*<0.05). The majority of the significantly differing abundant OTUs characteristic for captive cheetah faecal communities belonged to *Clostridium* cluster XIVa (5 OTUs) and *Clostridium* cluster XI (1 OTU). Faecal communities of domestic cats showed significantly higher abundance of 11 abundant OTUs (≥ 1% of reads) which were not detected in samples of captive cheetahs. These OTUs correspond to *Erysipelotrichaceae* (3 OTUs), *Veillonellaceae* (1 OTU), *Clostridium* cluster XIVa (3 OTUs), *Clostridium* cluster IV (1 OTU), *Coriobacteriaceae* (2 OTUs) and *Bifidobacteriaceae* (1 OTU). Especially OTUs belonging to the genera *Holdemanella*, *Catenibacterium* (*Erysipelotrichaceae*) and *Collinsella* (Coriobacteriaceae) were significantly more present in faecal communities of the 5 domestic cats (*P*=0.005) (**TABLE 7.1**). Most of the variation arose due to the

presence or absence of these variable OTUs in respective populations. Of note was the finding that cat 3 shared 30 OTUs (<0.5% of reads) with captive cheetahs that were not detected in any other cat included in this study. From these OTUs, 36% were taxonomically assigned to *Clostridium* cluster I and 33% to *Clostridium* cluster XI.

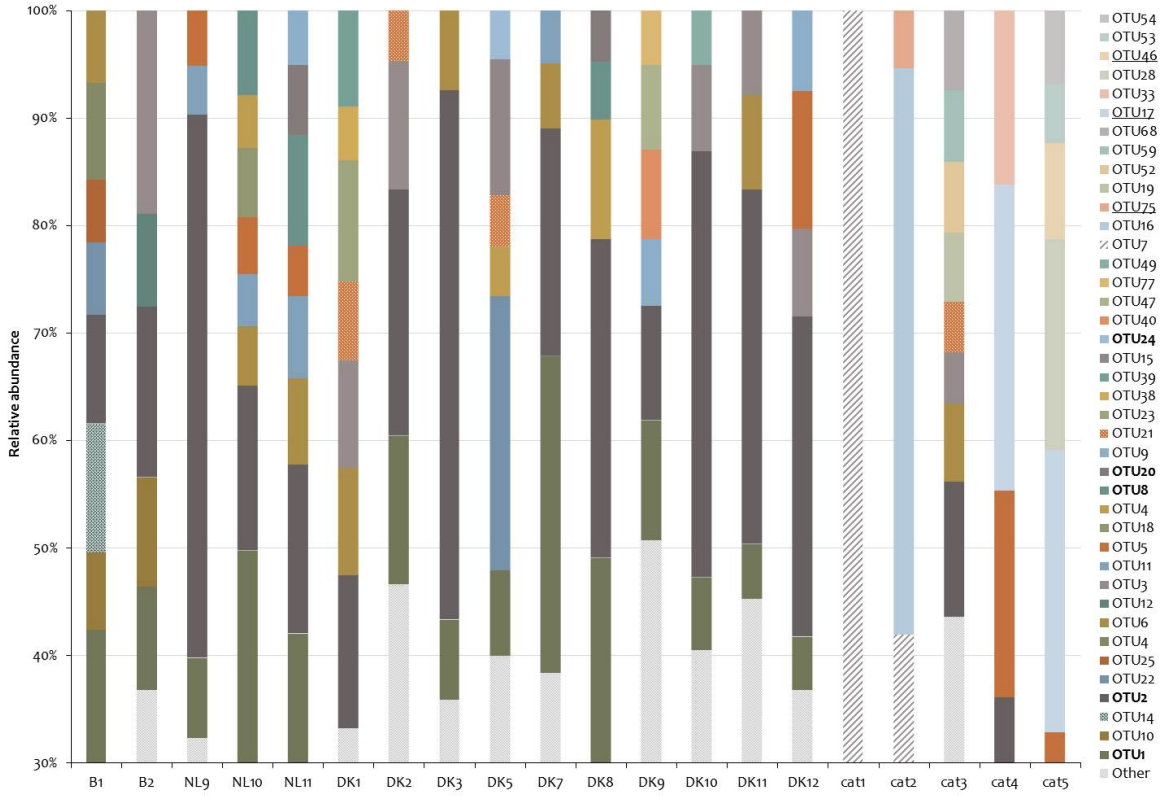


FIG 7.1 | Relative abundance of bacterial phylotypes (OTUs) across captive cheetah and domestic cat populations (enlargement from 30-100%) Bars show percentage of quality-controlled Illumina MiSeq reads classified to selected 99% OTUs for each library (= gut community of one animal). OTUs with a relative abundance <5% in an animal were lumped into a single category (i.e. other) for simplification. Origin is indicated beneath the animal IDs. OTUs significantly more present in cheetahs (bold) or cats (underlined) are indicated in the legend and derive from Kruskal-Wallis test (*P* adjusted by Benjamini-Hochberg correction<0.05).

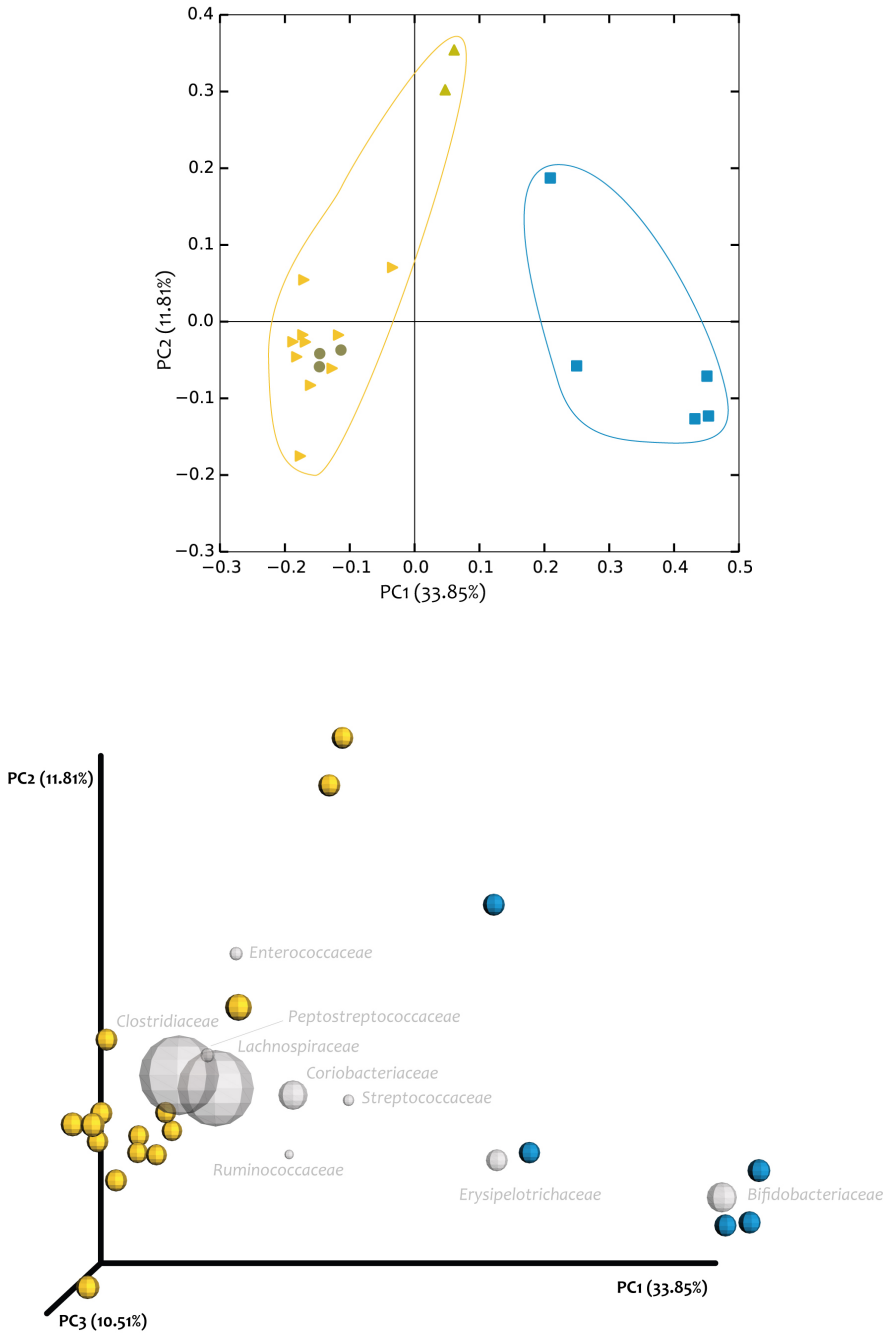


FIG 7.2 | PCA plot (a) and PCoA biplot (b) of unweighted UniFrac distances derived from Illumina data. All domestic cats (blue) and captive cheetahs (yellow) are included and different zoos are indicated by different colors and symbols (EB=yellow, OV=green, PL=light green). Superimposed are grey spheres indicating the most abundant bacterial families. The sizes of the spheres represent the mean relative abundance of the respective taxon and the location of the spheres within the plot indicate subject-specific associations.

TABLE 7.1 | Abundant OTUs (≥ 1% of reads in at least one sample) with significant different abundances between captive cheetah (n=15) and domestic cat (n=5) populations Kruskal-Wallis test (α=0.05) with Benjamini-Hochberg adjusted *P*-values.

OTU	*P* (adjusted)	Cheetah (mean # reads)	Cat (mean # reads)	Pylum	Class	Order	Family	Genus	Species	Closest Type Strain (%similarity)	*Clostridium* cluster
OTU2	0.032	15293	2597	Firmicutes	Clostridia	Clostridiales	*Clostridiaceae*	*Clostridium*	*hiranonis*	*Clostridium hiranonis* TO-931 (99.59)	XI
OTU1	0.021	7317	202	Firmicutes	Clostridia	Clostridiales	*Lachnospiraceae*			*Ruminococcus gnavus* ATCC 29149 (98.78)	XIVa
OTU8	0.021	2034	6	Firmicutes	Clostridia	Clostridiales	*Lachnospiraceae*	*Blautia*	*producta*	*Blautia hansenii* DSM 20583 (98.78)	XIVa
OTU20	0.021	1264	7	Firmicutes	Clostridia	Clostridiales	*Lachnospiraceae*	*Dorea*		*Ruminococcus faecis Eg2* (96.75)	XIVa
OTU24	0.025	683	0	Firmicutes	Clostridia	Clostridiales	*Lachnospiraceae*	*(Ruminococcus)*	*torques*	*Ruminococcus torques* ATCC 27756 (99.19)	XIVa
OTU32	0.021	672	0	Firmicutes	Clostridia	Clostridiales	*Lachnospiraceae*	*Dorea*		*Ruminococcus torques* ATCC 27756 (95.53)	XIVa
OTU46	0.020	0	1871	Firmicutes	Erysipelotrichia	Erysipelotrichales	*Erysipelotrichaceae*	*(Eubacterium)*	*biforme*	*Holdemanella biformis* DSM 3989 (100)	n/a
OTU17	0.005	0	6869	Firmicutes	Erysipelotrichia	Erysipelotrichales	*Erysipelotrichaceae*	*Catenibacterium*		*Catenibacterium mitsuokai* JCM 10609 (100)	n/a
OTU75	0.005	0	784	Firmicutes	Clostridia	Clostridiales	*Veillonellaceae*	*Megasphaera*		*Megasphaera elsdenii* DSM 20460 (100)	n/a
OTU83	0.005	0	407	Firmicutes	Clostridia	Clostridiales	*Lachnospiraceae*	*Dorea*		*Dorea formicigenerans* ATCC 27755 (97.56)	XIVa
OTU88	0.005	0	382	Actinobacteria	Coriobacteria	Coriobacteriales	*Coriobacteriaceae*	*Collinsella*		*Collinsella tanakaei* YIT 12063 (97.92)	n/a
OTU98	0.005	0	304	Actinobacteria	Coriobacteria	Coriobacteriales	*Coriobacteriaceae*	*Collinsella*		*Collinsella tanakaei* YIT 12063 (99.17)	n/a
OTU111	0.005	0	264	Firmicutes	Clostridia	Clostridiales	*Ruminococcaceae*			*Subdoligranulum variabile* DSM 15176 (99.59)	IV
OTU71	0.021	0	364	Firmicutes	Erysipelotrichia	Erysipelotrichales	*Erysipelotrichaceae*	*Bulleidia*	p-1630-c5	*Solobacterium moore* i JCM 10645 (93.62)	n/a
OTU87	0.021	0	350	Firmicutes	Clostridia	Clostridiales	*Lachnospiraceae*	*Blautia*		*Blautia wexlerae* WAL 14507 (96.75)	XIVa
OTU95	0.021	0	399	Firmicutes	Clostridia	Clostridiales	*Lachnospiraceae*	*Blautia*		*Blautia wexlerae* WAL 14507 (97.97)	XIVa
OTU97	0.021	0	470	Actinobacteria	Actinobacteria	Bifidobacteriales	*Bifidobacteriaceae*	*Bifidobacterium*	*longum*	*Bifidobacterium longum* subsp. *suis* LMG 21814 (100) *Bifidobacterium longum* subsp. *infantis* ATCC 15697 (100) *Bifidobacterium longum* subsp. *longum* JCM 1217 (100)	n/a

7.3.2 Variation of faecal communities across captive and free-ranging cheetah and domestic cat populations assessed with DGGE profiling and qPCR

The number of bands (apparent richness) in DGGE profiles of captive cheetahs (14.82 ± 0.56) was slightly higher compared to free-ranging cheetahs (12.00 ± 1.58) and domestic cats (10.00 ± 1.43). The mean similarity between DGGE profiles of samples from all captive cheetahs (n=50) was 61.75 ± 0.49% (range, 7.81 to 99.41%). No zoo-specific clustering was observed in DGGE clustering analysis. However, samples from captive cheetahs on a whole chicken diet grouped apart from cheetahs fed either a mixed prey and meat diet or a purely meat diet. In addition, DGGE profiles of these chicken-fed cheetahs clustered more closely to samples from free-ranging cheetahs than to those of other captive cheetahs (**FIG S7.3**). Intra-group similarity between DGGE profiles of samples from free-ranging cheetahs (n=8) was lower compared to captive cheetahs, *i.e.* 49.12 ± 4.53% (range, 0 to 79.24%). Lowest similarity was found between DGGE profiles of samples from domestic cats (mean 25.74 ± 5.91%; range, 0 to 65.10%). It is noteworthy that cat 3 did not cluster with other cats but fell into the cluster of captive cheetahs (**FIG S7.3).** Discriminative band-class analysis using a previously constructed reference framework from clone libraries indicated that the presence of Bcl 68.15 and 77.79 (assigned to *Clostridium* cluster I) and Bcl 75.34 (assigned to *Clostridium* cluster XI) discriminated cat 3 from its conspecifics. Also in cat 3, the band density of Bcl 80.93 which was taxonomically assigned to *Clostridium* cluster XI, was similar to the one found in band profiles of captive cheetahs but significantly higher compared to other cats ($P<0.05$). Overall, DGGE results for cat 3 confirmed the previous findings derived from Illumina MiSeq data.

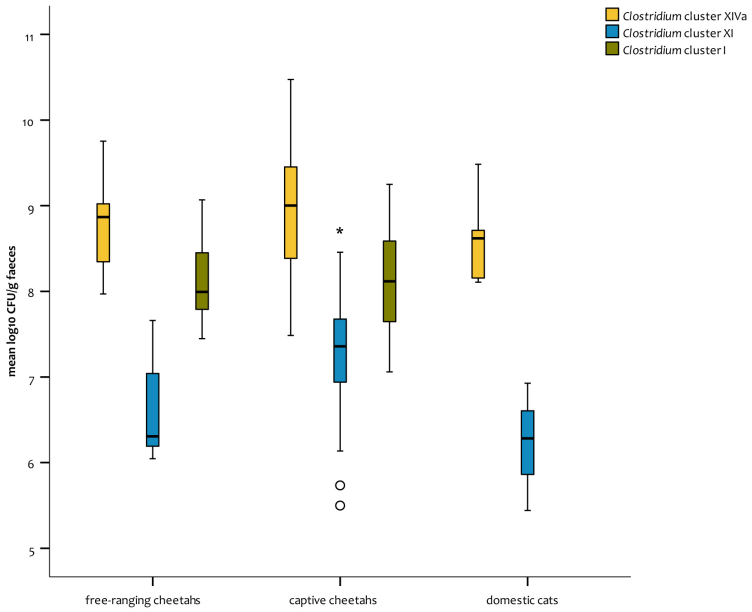


FIG 7.3 | Concentrations of *Clostridium* clusters XIVa, XI and I in faecal samples of domestic cats (n=5), free-ranging (n=7) and captive (n=50) cheetahs, assessed by real-time PCR Boxplots show median, interquartile range, sample minimum and maximum. The open circles indicate outlier values. Differences between populations in *Clostridium* cluster abundances were analysed using Kruskal-Wallis test (*$P<0.05$).

In qPCR, the Firmicutes to Bacteroidetes ratio showed a predominance of Firmicutes in free-ranging (1.16/0.04) and captive cheetahs (1.13/0.005) as well as in domestic cats (0.67/0.001). Samples of free-ranging cheetahs tended to hold relatively more members belonging to the phylum Bacteroidetes. Results from group-specific qPCR analyses (**FIG 7.3**) indicated that the abundance of *Clostridium* cluster XIVa in the faeces of free-ranging cheetahs (mean= 8.77 ± 0.20 log10 CFU/g) was not significantly different from captive cheetahs (mean= 8.97 ± 0.10 log10 CFU/g) and domestic cats (mean = 8.62 ± 0.23 log10 CFU/g). In contrast, *Clostridium* cluster XI was significantly more abundant in captive cheetahs (7.28 ± 0.09 log10 CFU/g) compared to free-ranging cheetahs (mean= 6.59 ± 0.21 log10 CFU/g) or domestic cats (mean= 6.22 ± 0.43 log10 CFU/g) ($P<0.05$). Clostridia of cluster I were abundant in the faeces of free-ranging (mean= 8.12 ± 0.18 log10 CFU/g) and captive cheetahs (mean= 8.13 ± 0.09 log10 CFU/g), but only present in low numbers (10^3 CFU/g faeces) or below detection limit in domestic cats. On the other hand, *Bifidobacterium* spp. were only detectable in low numbers ($\leq 10^4$ CFU/g faeces) or below detection limit in the majority of the cheetah samples, whereas they were present at 8.92 ± 0.94 log10 CFU/g in samples from domestic cats with the exception of samples from cat 3 in which *Bifidobacterium* spp. were not detected.

7.3.3 Compositional core assessment in captive cheetahs and domestic cats

OTUs and animal hosts were designated as nodes in a bipartite network, in which OTUs were connected to the animals in which their sequences were found (**FIG 7.4**). The network-based analyses showed that captive cheetahs shared more OTUs and thus a higher similarity between samples. In contrast, shared diversity among 15 cheetahs and 5 cats included only 7 OTUs. Of these core microbiota members, four OTUs were taxonomically assigned to *Clostridium* cluster XIVa, two to the *Coriobacteriaceae* and one to *Clostridium* cluster XI (**TABLE 7.2**). Accordingly, the most abundant signature taxa were retrieved in DGGE profiles through their high band density and presence in more than 80% of the samples, e.g. Bcl 48.97 affiliated to *Clostridium* cluster XIVa and Bcl 80.93 affiliated to *Clostridium* cluster XI.

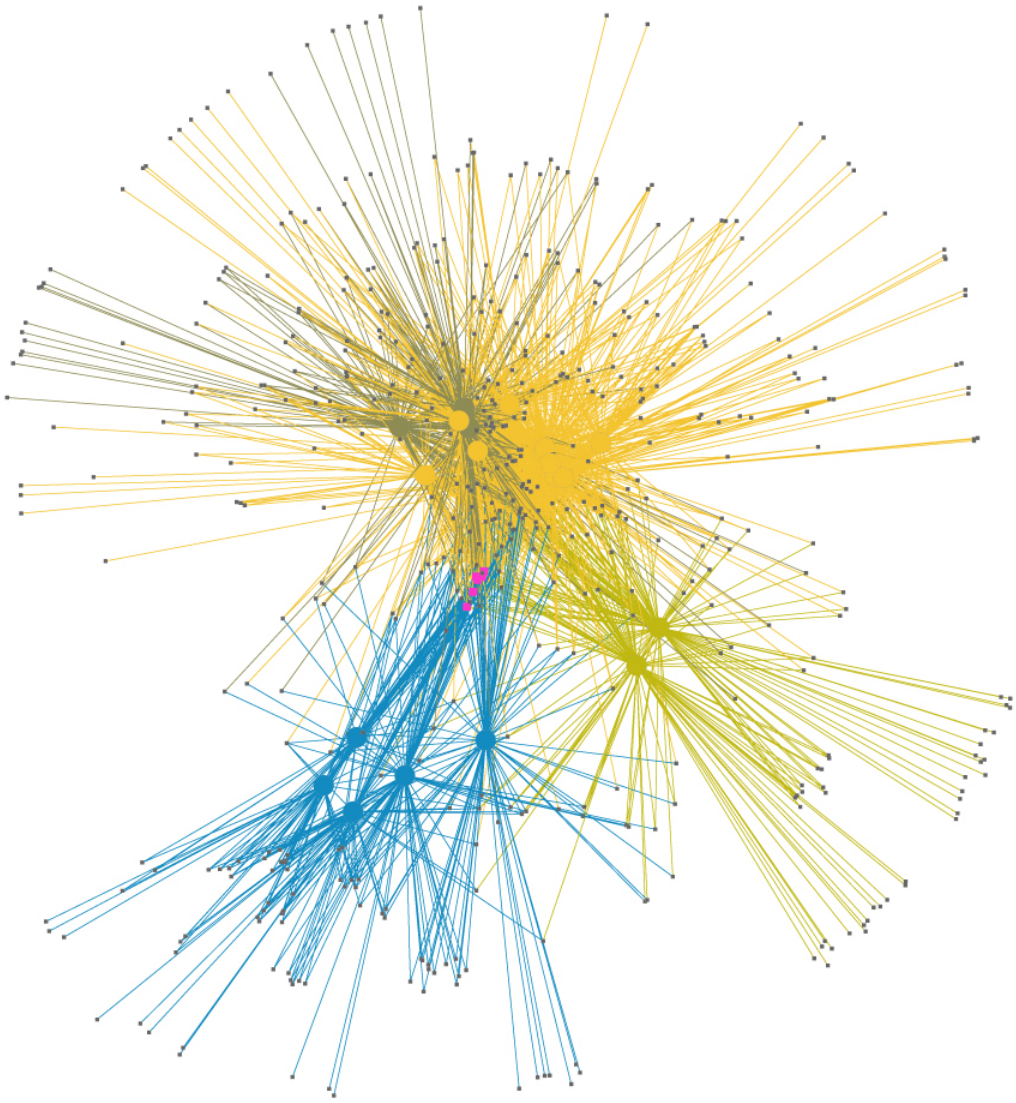


FIG 7.4 | Shared OTU diversity among faecal microbiota sampled from five domestic cats and 15 captive cheetahs
OTU network portrayed as spring-embedded layout, where OTUs that are in common bring nodes or samples together and OTUs that are distinct repel samples. Network edges and sample nodes (round) are colored by host (cat=blue, cheetah=yellow/green) and origin (EB=yellow, OV=green, PL=light green). Black squares represent OTUs, whereas pink squares indicate shared OTUs between all samples.

TABLE 7.2 | Shared OTUs between 15 captive cheetahs and 5 domestic cats, their taxonomic assignment at family level and closest type strain.

OTU[a]	relative abundance (%)		Phylum	Order	Family	*Clostridium* cluster	Closest type strain (% sequence similarity)
	cheetahs (885502 reads)	cats (298561 reads)					
OTU1	12.23 ± 2.04	0.36 ± 0.24	Firmicutes	Clostridiales	*Lachnospiraceae*	XIVa	*Ruminococcus gnavus* ATCC 29149^T (98.78)
OTU2	25.56 ± 3.38	4.64 ± 2.37	Firmicutes	Clostridiales	*Peptostreptococcaceae*	XI	*Clostridium hiranonis* TO-931^T (99.59)
OTU4	3.57 ± 0.77	0.85 ± 0.49	Firmicutes	Clostridiales	*Lachnospiraceae*	XIVa	*Blautia glucerasea* HFTH-1^T (99.18)
OTU5	2.80 ± 0.84	6.40 ± 3.49	Actinobacteria	Coriobacteriales	*Coriobacteriaceae*	n/a	*Collinsella aerofaciens* JCM 10188^T (99.58)
OTU6	4.73 ± 0.77	1.85 ± 1.38	Firmicutes	Clostridiales	*Lachnospiraceae*	XIVa	*Blautia obeum* DSM 10518^T (95.93)
OTU9	2.77 ± 0.58	1.30 ± 0.78	Firmicutes	Clostridiales	*Lachnospiraceae*	XIVa	*Blautia glucerasea* HFTH-1^T (94.69)
OTU11	2.51 ± 0.56	0.90 ± 0.52	Actinobacteria	Coriobacteriales	*Coriobacteriaceae*	n/a	*Collinsella intestinalis* DSM 13280 (100)

[a]OTUs delineated at 99%; OTU = operational taxonomic unit; n/a = not applicable

7.4 Discussion

Gut-associated microbial communities are thought to have co-evolved with their mammalian hosts towards a tight symbiotic relationship [86,429]. Exploring the gut microbial composition of a broad range of captive cheetahs, subject to distinct environmental factors, and comparison of their gut microbiota with that of free-ranging conspecifics thus makes an interesting case to explore if there is a 'core' set of gut microbial lineages shared by these strict carnivores classified in the *Felidae* (Carnivora).

The present study includes the largest population of captive cheetahs ever studied for host microbiota profiling. DGGE-based community fingerprinting revealed a remarkably high level of similarity (61.75 ± 0.49%) between the faecal microbiota of 50 captive cheetahs housed in 13 different zoos, and did not show a significant grouping of animals according to zoo, sex or age category. Also at a finer taxonomic level, Illumina sequencing revealed that a subset of 15 captive cheetahs housed in 3 different zoos shared 73.3% of the reads representing 3.4% of OTUs (15 OTUs) of all OTUs. The majority of these affiliated to *Clostridium* cluster XIVa. Taken together with the high abundance of *Clostridium* cluster XIVa (mean= 8.97 log10 CFU/g) in all captive cheetah samples revealed by qPCR analysis, these findings corroborate earlier observations that this group constitutes one of the most numerically predominant strict anaerobes in faecal samples from captive cheetahs based on 16S rRNA gene clone libraries (43%) [351] and Illumina MiSeq sequencing (20%) [428]. Upon comparison with free-ranging conspecifics, members of *Clostridium* cluster XIVa were equally abundant (mean= 8.77 log10 CFU/g), and also overall community richness was not significantly different between free-ranging and captive cheetahs. This is in contrast to other animals such as Howler monkeys and Capercaillie birds for which a depletion of gut microbial diversity has been reported in captive conspecifics [423,425]. Conversely, an increased OTU richness was reported in the gut microbiota of captive leopard seals compared to wild conspecifics [378]. From these previous studies and our findings for cheetahs, it can be hypothesized that identity of the animal host and its environment play a role in the way faecal microbiota of conspecifics may diverge. Indeed, the external environment of captive hosts and their free-ranging conspecifics can differ considerably (e.g. climate, co-habitation, interaction with humans, dietary patterns), all of which may influence metabolic rate, body condition, hormonal production and other factors [29,430] shaping the microbial composition and/or functional capacity of the gut [431–433]. Recent data in wild-caught rodents suggest that as animals enter captivity, they retain the unique microbial community harboured in the wild with relative abundances of some species shifting over time [434]. The significantly higher proportion of *Clostridium* cluster XI in captive cheetahs compared to free-ranging conspecifics might substantiate this hypothesis. *Clostridium* cluster XI are typically enriched in the gut microbiota of carnivores and include both proteolytic as well as toxinogenic clostridia. A higher proportion of *Clostridium* cluster XI has also been found in captive grizzly bears compared to their wild counterparts, but did not coincide with increased enteric pathogen load [265]. In this context, samples from captive

cheetahs could be further tested for genes encoding the phospholipase C cspC of *Clostridium sordellii* or *C. difficile* toxin B tcdB. Although only one case of *C. sordellii* infection has been reported in captive lions [435] and *C. difficile* infections are rarely reported in felines [436], cheetahs housed in zoos may still be potential reservoirs of these enteropathogens for humans or other animals.

Whereas zoo location, sex and age category did not contribute to differential clustering among captive cheetahs, our study revealed some evidence that diet composition and regimen may trigger the divergence of faecal communities. The observation that captive cheetahs fed only whole chickens clustered together and more closely resembled profiles of free-ranging cheetahs may stem from the fact that a changed supply of particular prey components (e.g. feathers, viscera) can result in changes in substrate availability for specific gut members [378]. Cheetahs initially target muscle meat and possibly some internal organs from large prey species, whereas the consumption of whole rodents, small mammals or birds is likely to provide other parts of prey such as intestines, fur, skin, bones or feathers [19]. However, it is not entirely clear whether this is only a signature of dietary relatedness. Moreover, the extent to which the free-ranging cheetahs under study include small prey in their diet compared to larger kills is unkown and requires thorough long-term monitoring.

An extensive study of the diversity of the microbiomes in 59 mammalian species showed that both phylogeny and diet are driving factors of gut microbial composition [86]. To this end, microbiome data for captive and free-ranging cheetahs should be mirrored to data from other *Felidae*, and may further provide information on the potential impact of different dietary habits on gut microbial community composition within feline hosts. Domestic cats present an interesting case for comparison given the fact that this frequently used model for strict carnivorism is mostly fed commercial petfoods, which do not resemble the natural diet of a strict carnivore. Although the cat samples included in this study are limited in number, the significantly different gut microbial communities revealed between captive cheetahs and domestic cats with Illumina MiSeq sequencing might hint to future research directions. In accordance with our previous studies [351], *Bifidobacterium* spp. were retrieved from faecal samples from domestic cats but rarely from cheetah faecal samples. In addition, faecal samples from cats included in this study were dominated by *Erysipelotrichaceae* (*Catenibacterium* spp.) and *Veillonellaceae* (*Megasphaera* spp.). These specific genera are lower in kittens fed high-protein diets compared to kittens fed moderate-protein diets [296] and in cats fed commercial wet diets compared to dry diets [298]. The remarkably different gut bacterial composition between domestic cats and captive cheetahs supports the hypothesis that long-term dietary adaptions, in this case to commercial petfoods such as dry kibble diets, might alter gut microbial composition. This hypothesis is supported by the divergent faecal microbial composition of cat 3, which was fed a home-prepared raw meat diet more similar to the dietary pattern of captive cheetahs. This cat had higher proportions of OTUs relating to *Clostridium* cluster XI and shared a number of OTUs with captive cheetahs which were not detected in the other domestic cats. Similarly, the faecal microbiota of domestic cats fed raw whole chicks was different from

cats fed an extruded chicken-based diet [437]. To this end, exploring the faecal community composition in wild, feral cats that typically eat small prey and insects and whose diet contains 63% crude protein on a dry matter basis [375] might provide valuable information.

Although captive cheetahs and domestic cats shared 17 out of 28 identified bacterial families, relative abundances varied greatly between both feline host species. At a finer taxonomic level, 7 shared OTUs taxonomically assigned to *Clostridium* cluster XIVa, *Clostridium* cluster XI and the *Coriobacteriaceae* contributed to only 16.3% of reads in cat samples compared to 54.17% of reads in cheetah samples. Type strains most closely affiliating to the four common OTUs within *Clostridium* cluster XIVa included different species of *Blautia*, a genus that was previously found in higher proportions in cat samples (5.8%) compared to humans (1.2%) and dogs (1.3%) based on oligotyping of faecal microbiota [438]. Moreover, *Blautia* oligotypes could accurately identify different hosts across different dietary patterns, suggesting that host physiology may be a major determinant in the finer architecture of *Blautia* populations [438]. This specific population structure in a single genus member of *Clostridium* cluster XIVa likely represents a pool of metabolic capacity optimized for a host. Further identification of host-preference patterns of genera and species within *Clostridium* cluster XIVa might thus provide new information about the drivers of adaptation between bacterial organisms and their hosts and which of the many functions related to *Clostridium* cluster XIVa [439] are carried out in the specific hosts.

7.5 Conclusion

The study of the nature and extent of the feline core and non-core taxa provides a framework for comparing hosts across time and habitat, and for further understanding the roles that evolutionary and ecological processes may play in shaping the microbial patterns in *Felidae*. The data from our exploratory study provide some insight into future directions of feline microbiome research. Although diet appears to be a key driver, additional studies are needed to also sort out multifactorial interactions with host genetic and environmental influences. Also, comparisons between faecal microbiota of wild and captive cheetahs should investigate changes in microbial gene expression which may exhibit larger changes and greatly impact host physiology. Finally, comparative studies including different members of the *Felidae* will reveal to what extent the domestic cat can or cannot serve as a model for feline species.

SUPPLEMENTARY DATA

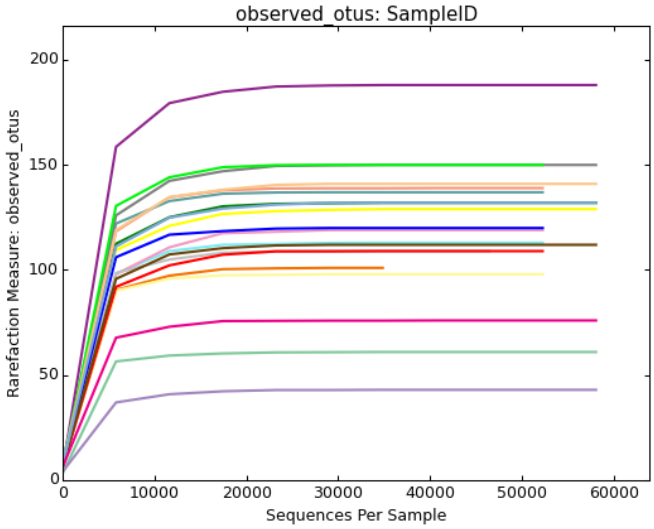


FIG S7.1 | Rarefaction curves of microbial communities from 15 samples from captive cheetahs and 5 samples from domestic cats. Cat 1 (light green), cat 2 (light purple), cat 3 (yellow), cat 4 (grey) and cat 5 (pink); other colors for cheetahs

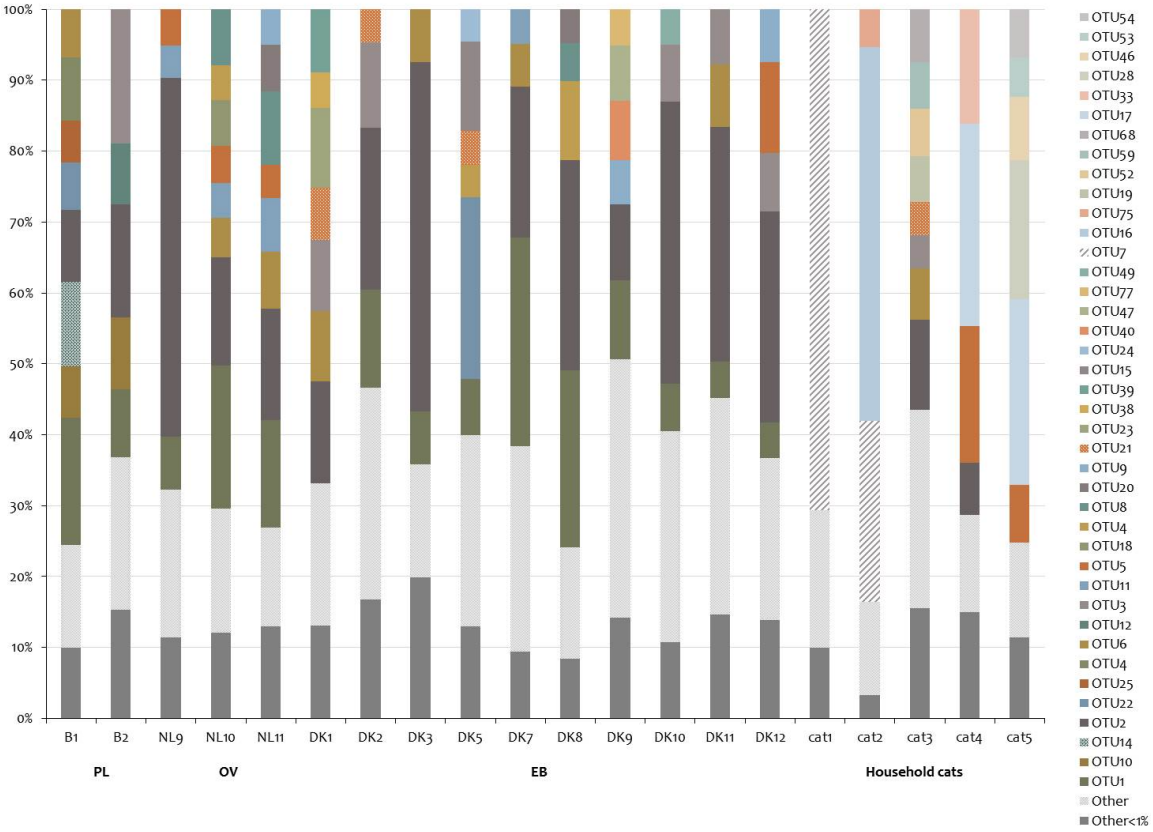


FIG S7.2 | Relative abundance of bacterial phylotypes (OTUs) across captive cheetah and domestic cat populations. Bars show percentage of quality-controlled Illumina MiSeq reads classified to selected 99% OTUs for each library (gut community of one animal). OTUs with a relative abundance <5% in an animal were lumped into a single category (i.e. other) for simplification. Origin is indicated beneath the animal IDs. OTUs significantly more present in cheetahs (bold) or cats (underlined) are indicated in the legend and derive from Kruskal-Wallis test (*P* adjusted by Benjamini-Hochberg correction<0.05)

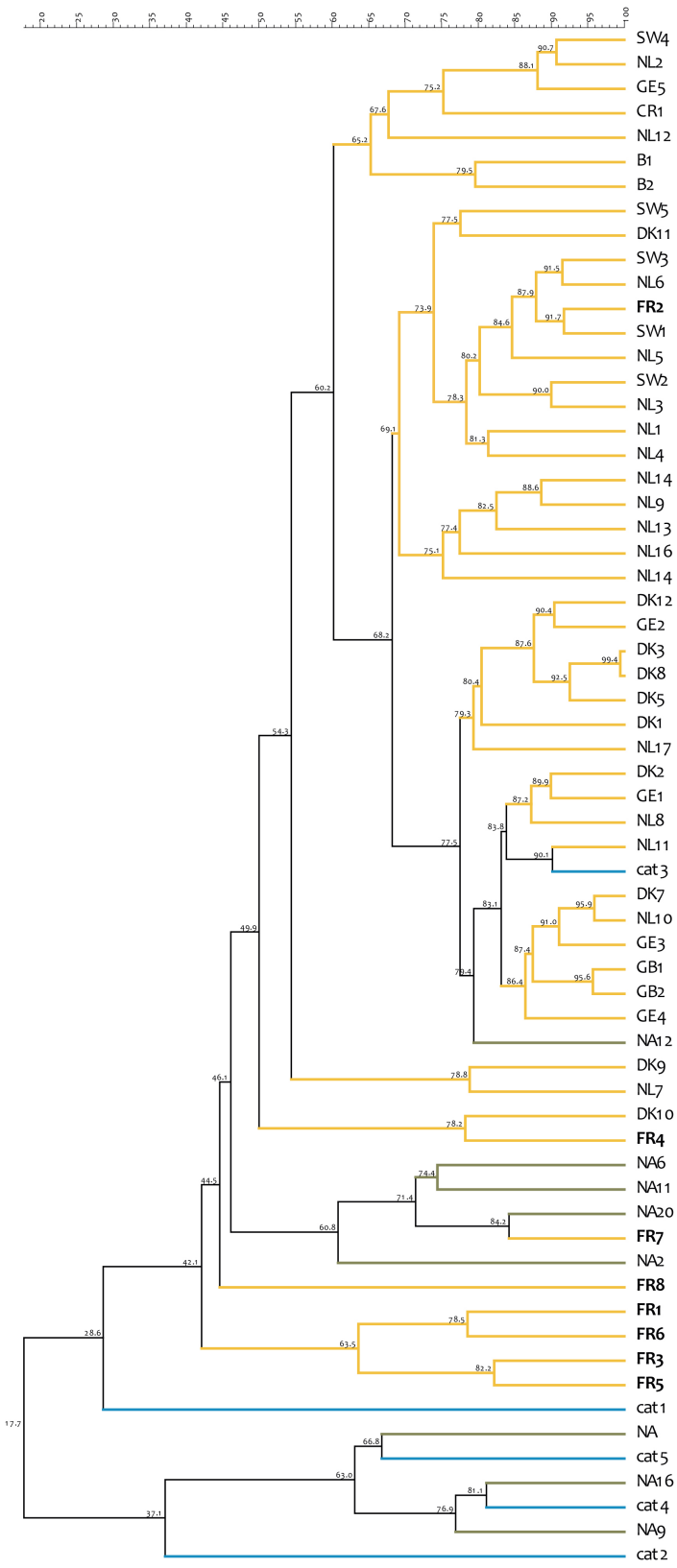


FIG S7.3 | Rooted UPGMA dendrogram based on Pearson correlation coefficient matrix depicting the similarities between DGGE community profiles of faecal samples collected from 50 different captive cheetahs, 8 free-ranging cheetahs and 5 domestic cats. Samples are represented by the name of the animal and coloured by origin (captive cheetahs = yellow, free-ranging cheetahs = green, domestic cats = blue). Samples from cheetahs on a strict whole chicken diet are indicated in bold.

TABLE S7.1 | Sample metadata for the samples included in this study.

Country	Zoo	Dietary regime at the time of collection (all include 1 fasting day/week)	Animal	Age (y)	Sex	Sibling	Co-housing	Techniques for this study
Belgium	Zoo Parc Planckendael (PL)	2 kg chunked horsemeat*/animal/day	B1	9	M	B2 (twin)	B1 & B2	a;b
		randomly interspersed with 1 kg whole rabbit (4x/month)	B2	9	M	B1 (twin)		a;b
Croatia	Zagreb Zoo (ZZ)	1.5 - 2 kg whole rabbit/animal/day (no fasting days)	CR1	12	M			a;b
Denmark	Reepark Ebeltoft Safari (EB)	1-2 kg whole rabbit or chicken*/animal/day; randomly interspersed with 1-1.5 kg chunked beefmeat*/animal/day (3x/month)	DK1	5	M			a;b
			DK2	2	F	DK3 (twin)	DK2 & DK3	a;b
			DK3	2	F	DK2 (twin)		a;b
			DK5	7	F			a;b
			DK7	4	F			a;b
			DK9	5	F		DK9 & DK10 & DK11	a;b
			DK10	1	M	DK9 (mother); DK12 (father); DK11 (twin)		a;b
			DK11	1	M	DK9 (mother); DK12 (father); DK10 (twin)		a;b
			DK12	9	M	DK10 & DK11 (sons)		a;b
France	Réserve Africaine de Sigean (SI)	1 whole chicken/animal/day	FR1	2	M	FR6 (twin)		b
			FR2	3	F			b
			FR3	13	F			b
			FR4	4	M	FR5 (twin)		b
			FR5	4	F	FR4 (twin)		b
			FR6	2	M	FR1 (twin)		b
	Zoo de Montpellier (MO)	1 whole chicken/animal/day	FR7	2	M			b
			FR8	2	M			b
Germany	Wilhelma (WI)	chunked horsemeat with bone	GE1	8	M			b
	Zoologisch-Botanischer Garten Stuttgart	cooked chicken without skin, cooked rice and minced horsemeat	GE2	10	F			b
	Zoo Parc Erfurt (ER)	chunked beefmeat*	GE3	2	F			b
			GE4	4	M			b
	Allwetterzoo Münster (MU)	1 whole rabbit/animal/day (3x/week); 1 chicken/animal/day (1x/week); beef- or horsemeat (2x/week)	GE5	6	F			b
The Netherlands	Safaripark Beekse Bergen (BB)	chunked beefmeat*, oft with bone; randomly interspersed with 1kg whole rabbit (1x/week)	NL1	3	F			b
			NL2	5	M			b
			NL3	10	F	NL4 (daughter)		b
			NL4	2	F	NL3 (mother)		b
			NL5	9	F			b
			NL6	9	F			b
			NL7	5	F	NL3 (mother); NL4 (half sister)		b
			NL8	2	F			b
			NL16	2	F			b
			NL17	10	F			b
	Zoo Parc Overloon (OV)	1 whole rabbit/animal/day; regularly interspersed with chunked beef carcasses*; sometimes chickens and chunks of antelope carcasses*	NL9	11	M		NL9 & NL10	a;b
			NL10	9	M			a;b
			NL11	6	F			a;b
			NL12	5	M			b
	Gaia Zoo (GA)	2x/day: 250 g minced meat + 250 g chopped meat + 90 g Royal Canin Renal kibble + 100 mL water + Pancrex and 20 mg pill Prineral (only in the morning)	NL13	12	M	NL14 & NL15 (twins)	NL13 & NL14 & NL15	b
		20 mg pill Prineral with some meat (only in the morning); 1.5 kg chunked horsemeat + 5g Ipakitine + 2 g Gistocal + 200 mL water with blood	NL14	12	M	NL13 & NL15 (twins)		b
		1.4 kg chunked horsemeat + 2 g Gistocal + 200 mL water with blood	NL15	12	M	NL13 & NL14 (twins)		b
Sweden	Boras Djurpark (BO)	1.5-2 kg chunks of beef or horse with bone*/animal/day (no fasting days); small pieces of heart and liver; special diet SW1: rabbit, eland antelope, sheep or chicken (no beef or horse) with extra fibre: Visiblin (6g/meal)	SW1	8	M			b
			SW2	1	M	quintuplet	5 brothers	b
			SW3	1	F	twins	2 sisters	b
			SW4	1	F		Mother + 2 daughters	b
			SW5	1	M	triples	3 brothers	b
United Kingdom	Colchester Zoo (CO)	chunked beefmeat	GB1	7	M			b
		randomly interspersed with 1 kg whole rabbit (1x/week)	GB2	5	F			b
Namibia	Cheetah Conservation Fund (CCF)	hunting prey (e.g. antelopes, wildebeests, birds,...) in their natural habitat	NA2	-	M		free-ranging	b
			NA6	-	M			b
			NA9	-	F			b
			NA11	-	M			b
			NA12	-	M			b
			NA16	-	F			b
			NA18	-	F			b
			NA20	-	M			

note: whole prey include fur, feather, skin, bones, rabbit head but no chicken head and sometimes viscera unless otherwise stated

TABLE S7.2 | Statistics of Illumina MiSeq data generated for 15 faecal samples from captive cheetahs housed at Zoo Parc Planckendael (PL, Belgium), Zoo Parc Overloon (OV, the Netherlands) and Reepark Ebeltoft Safari (EB, Denmark) and 5 faecal samples from 5 domestic cats housed at 5 different households.

Location	Animal	# clean reads	Shannon Diversity Index	Gini Index	# OTUs
PL	B1	53754	4.12	0.97	109
	B2	56501	4.32	0.96	120
OV	NL9	55608	3.29	0.97	139
	NL10	64304	4.29	0.97	132
	NL11	58979	4.30	0.97	141
EB	DK1	40519	4.44	0.96	101
	DK2	73003	4.43	0.96	132
	DK3	65692	3.65	0.96	188
	DK5	70136	4.27	0.97	129
	DK7	56777	3.79	0.97	113
	DK8	57622	3.43	0.98	119
	DK9	54124	5.01	0.95	137
	DK10	58486	3.79	0.97	112
	DK11	58979	4.23	0.96	150
	DK12	55873	4.21	0.96	150
Household	cat1	66936	2.22	0.99	61
	cat2	58976	2.19	0.99	43
	cat3	56531	4.89	0.95	98
	cat4	50822	3.72	0.97	109
	cat5	65296	3.74	0.98	76

In preparation:

Becker AAMJ, Hesta M, Van de Wiele T, Vanden Bussche J, Vanhaecke L, Janssens GPJ, Huys G. Adapting a dynamic simulator of the human intestinal microbial ecosystem to an *in vitro* model mimicking the microbial composition and metabolic homeostasis within the gut environment of the cheetah, a strict carnivore with vulnerable status.

8 ADAPTING A DYNAMIC SIMULATOR OF THE HUMAN INTESTINAL MICROBIAL ECOSYSTEM TO AN *IN VITRO* MODEL MIMICKING THE MICROBIAL COMPOSITION AND METABOLIC HOMEOSTASIS WITHIN THE GUT ENVIRONMENT OF THE CHEETAH, A STRICT CARNIVORE WITH VULNERABLE STATUS

Dynamic *in vitro* gastrointestinal simulation models provide a useful platform to study mechanistic effects of dietary or therapeutic interventions on microbial dynamics and metabolic homeostasis in the human gut. Also for mammalians with vulnerable status and limited sampling access, such models could offer a tool for reproducible and non-invasive analyses and monitoring of intestinal processes that may have a potential impact on host health and, ultimately, conservation of the species. This study sets the stage for the adaptation of the well-validated Simulator of the Human Microbial Ecosystem (SHIME) to a dynamic feline gut model mimicking the gastrointestinal tract of the strictly carnivorous cheetah (*Acinonyx jubatus*) under carbohydrate-depleted protein-rich conditions. Essentially, the model consists of a three-stage sequential reactor system inoculated with fresh cheetah faeces from which effluents were subjected to SCFA and ammonium analyses, microbial community fingerprinting and untargeted metabolic fingerprinting. Under adapted nutritional and physiological conditions that approached those of the cheetah gut, microbial communities reached compositional and functional stability after two weeks and this steady-state was reproducible between nine parallel reactors. Upon stabilization, we monitored the fermentation potency in the simulated cheetah gut by challenging it for a 10-day period with hydrolyzed and non-hydrolyzed forms of collagen, a typical constituent of the strict carnivore's diet. Control reactors without substrate supplementation maintained microbial and metabolic stability over 30 days, indicating the model's capacity to continue a long-term steady-state. Fermentation of hydrolyzed collagen peptides resulted in modification of the microbial and metabolite signatures, whereas non-hydrolyzed collagen did not significantly alter microbial or metabolite profiles. Yet, enzymatic predigestion of non-hydrolyzed collagen resulted in increased levels of hydroxyproline as a marker of collagen degradation as well as an increase in total SCFA. Only 8% of signature metabolites could be unambiguously identified. Although additional finetuning is required, we anticipate that the SHIME model adapted 'from man to cheetah' can be used for *in vitro* simulations of dietary interventions in carnivores and the analytical assessment of their compositional and functional consequences.

8.1 Background

Given the high physiological, microbial and metabolic complexity of the mammalian gut environment and its difficult direct access [122], *in vitro* fermentation models mimicking microbial processes along the gastrointestinal tract (GIT) represent enticing alternatives over *in vivo* models. They are typically cheaper, offer flexibility and scalability for hypothesis testing, allow dynamic sampling in different intestinal regions and are able to provide reproducible results due to standardization and strict control of environmental factors such as retention time, nutrient intake, pH and temperature. In addition, there are no critical ethical constraints for using *in vitro* models [230,231,408]. While the use of different luminal [230,231] and mucosal [440] *in vitro* gut models gained mechanistic insights in the functional and ecological role of the human colon microbiota, optimized *in vitro* models to study intestinal microbial activities of other mammalian hosts are rare and usually restricted to simple batch fermentations. Only recently, long-term continuous *in vitro* models dedicated to the pig (PolyfermS model) [441] and dog GIT (SCIME^{TM}, Prodigest, Belgium) started to make their way into the field.

The adaptation or development of fermentation models for specific food producing or companion animals creates new opportunities for the feed and petfood industry to study in detail the effect of macronutrient composition, functional ingredients and fibre content on gut microbial communities. For example, recent *in vivo* studies have shown that dietary interventions in cats (e.g. high-protein versus low-protein diets, inclusion of beet pulp, pectin or cellulose as fibre source, and comparison of different protein:fat:carbohydrate ratios) have measurable effects on microbial composition and activity, with potential relevance to gut health (reviewed in [261]). However, most of these studies have only considered faecal samples as a proxy for distal colon communities and information on functional microbial potential has been mainly hypothesized from sequenced bacterial genomes [286,297]. In this regard, cause-and-effect relationships remain unclear and warrant integration of multi-omics data and *in vitro* techniques to better understand and predict the functional potential of the hitherto underexplored gut microbial communities in carnivores and their implications for host health [222,261].

Members of the mammalian family *Felidae* have evolved as strict carnivores, and rely on protein-rich animal tissues to meet their unique nutritional requirements [249,442]. However, whereas domestic cats might have adapted to commercial petfoods containing variable amounts of carbohydrates and fibres, most exotic felids housed in zoos are fed raw meat diets or whole prey diets depending on availability. Amongst others, appropriate nutrition is considered one of the key elements in animal health and welfare, and therefore has consequences for *ex situ* conservation programmes for cheetahs and other endangered strict carnivores [443]. Given the limited sampling access typical for animals with vulnerable status, *in vitro* models may thus be a valid approach to study those parameters that have a potential impact on digestion and host health and consequently specify targets for and reduce the number of subsequent *in vivo* trials.

In this study, we set out to adapt the Simulator of the Human Intestinal Microbial Ecosystem (SHIME; registered trademark, Ghent University and ProDigest, Belgium) [444] towards a model that mimics the intestinal microbial composition and stability found in the cheetah as previously described for captive animals [351,401]. The aims of our approach were (i) to adapt the SHIME model towards a dynamic *in vitro* gut model that mimics the cheetah GIT, (ii) to achieve a steady-state in terms of microbial community composition and metabolic activity in the dynamic *in vitro* gut model mimicking the cheetah GIT and (iii) to evaluate the potential of this *in vitro* gut model to study the ecological impact of dietary interventions using non-hydrolyzed and hydrolyzed collagen as test substrates. For these purposes, model parameters were adjusted to mimic the cheetah's GIT physiology and the nutritional medium used in the *in vitro* gut model was reformulated to simulate a strict carnivore's diet in a series of preliminary batch incubations. Conventional metabolic analyses were combined with microbial community fingerprinting and a high-throughput high-resolution mass spectrometry-based untargeted metabolomics approach [445] to evaluate microbial community composition and metabolic activity throughout the different adaptation and intervention steps.

8.2 Material & Methods

Chemicals and preparation of nutritional media

Unless stated otherwise, all chemicals were obtained from Sigma (Diegem, Belgium).

The static batch incubations were conducted using a sugar-depleted, phosphate buffered medium (pH 5.9; 0.1 M) containing 12.25 g/L KH_2PO_4, 1.78 g/L Na_2HPO_4, 3 g/L yeast extract, 4 g/L mucin from porcine stomach Type II and 10 g/L proteose peptone (LP0085, Oxoid) or meat peptone (Fluka Sigma 70174, peptic digest from bovine meat). The medium used in the dynamic gut model (*i.e.* feed) was a sugar-depleted formulation containing 12.25 g/L KH_2PO_4, 1.78 g/L Na_2HPO_4, 1.65 g/L yeast extract, 2.2 g/L mucin and 5.5 g/L meat peptone (Fluka Sigma 70174). The pancreatic juice contained 0.5 g/L pancreatin, 6.88 g/L $NaHCO_3$ and 3.33 g/L bile salts (Difco). Specific substrates used in this study include non-hydrolyzed collagen type I from bovine achilles tendon (C9879, Sigma) and peptides from bovine hydrolyzed collagen (Peptan B 2000 LD, Rousselot, Ghent, Belgium). Type 1 collagen fibres are the main component of connective tissues and make up to 30% of total proteins in mammallian bodies, consequently being a major component of a strict carnivore's diet. Pure trans-4-Hydroxy-L-proline (≥ 99%) (56250, Sigma) was included for targeted metabolite profiling.

Donor and faecal sample preparation

For batch experiment I fresh faecal samples were collected immediately upon defaecation from an adult male cheetah (NL12; 4 years old) housed at Zoo Parc Overloon (OV; Overloon, the Netherlands), a full member of the European Association of Zoos and Aquaria (EAZA, http://www.eaza.net/membership). The animal shared indoor and outdoor housing with another 4-year old adult male cheetah (NL18) from whom samples were collected for batch experiment II and for optimization of the dynamic *in vitro* gut

model. Both animals were fed their regular zoo diet consisting of unsupplemented whole rabbits randomly interspersed with chicken, vitamin and mineral supplemented chunked boneless horsemeat (1.5 kg/day/animal) or pieces of beef, horse and antelope carcasses. Both cheetahs were treated prophylactically on a three monthly basis for internal parasites (Drontal Cat; Bayer, Mijdrecht, the Netherlands) and had no history of gastrointestinal disorders. No medical or health problems were apparent on remote examination.

Fresh faecal samples were immediately collected upon defaecation in a closed recipient that was maintained anaerobically by adding AnaeroGen^{TM} sachets (Oxoid, Hampshir, UK) and transported at 4°C. Within 4h after collection, 40 g of the freshly voided faecal sample was homogenised and diluted with 200 mL 0.1 M anaerobic potassium phosphate buffer (8.8 g/L K_2HPO_4, 6.8 g/L KH_2PO_4, pH 7.0) containing 1 g/L sodium thioglycolate as reducing agent. After removal of the particulate material by mild centrifugation (5 min at 500g), the supernatant was used as inoculum.

Experimental setup

Static short-term batch fermentations

Two batch fermentation experiments (*i.e.* experiment I and II) were performed and the different test conditions for each of them are summarized in **TABLE 8.1**. In batch experiment I, the fermentation of proteose peptone, peptic digested meat peptone or a mixture thereof was evaluated in short-term (24h) static batch incubations inoculated with cheetah faecal microbiota. Proteose peptone is a standard component of the SHIME feed [446] and consists of a mixture of meat-, vegetable- and casein-derived peptones, whereas peptic digested meat peptone only supplies meat-derived peptones. Compared to the conventional SHIME feed, the polysaccharides and starch components were omitted, the amount of yeast extract and mucin remained the same and the amount of the peptone component was increased from 1 g/L to 10 g/L. Each condition was tested in quadruplicate.

TABLE 8.1. | Summary of test conditions in two short-term (24h) batch fermentations conducted with cheetah faecal inoculum at 38.5°C

Short-term batch incubations (24h)	Test conditions (# of replicates)	Composition of the nutritional medium (32 mL/bottle)[a]
Batch experiment I	M1 (A, B, C, D)	10 g/L proteose peptone
	M2 (A, B, C, D)	5 g/L proteose peptone + 5 g/L meat peptone
	M3 (A, B, C, D)	3 g/L proteose peptone + 7 g/L meat peptone
	M4 (A, B, C, D	10 g/L meat peptone
Batch experiment II	F (A, B, C)	10 g/L meat peptone
	FsCo (A, B, C)	10 g/L meat peptone + 0.5 g collagen
	FlCo (A, B, C)	10 g/L meat peptone + 1 g collagen
	FsCop (A, B, C)[b]	10 g/L meat peptone + 0.5 g collagen
	FlCop (A, B, C)[b]	10 g/L meat peptone + 1 g collagen
	FsPe (A, B, C)	10 g/L meat peptone + 0.5 g Peptan B
	FlPe (A, B, C)	10 g/L meat peptone + 1 g Peptan B

[a] non-variable medium components: 12.25 g/L KH_2PO_4, 1.78 g/L Na_2HPO_4, 3 g/L yeast extract, 4 g/L mucin from porcine stomach Type II

[b] pre-digestion step: acidification of medium during 1h (pH= 2.5) and then 1.5h incubation with pancreatic juice (13.7 mL/bottle)

In batch experiment II, the fermentability of non-hydrolyzed collagen type I and hydrolyzed collagen type I (further referred to as Peptan B) were investigated in short-term (24h) static batch incubations inoculated with cheetah faecal microbiota (**TABLE 8.1**). The addition of different substrate amounts was evaluated alongside a control without substrates. Also the inclusion of a pancreatic digestion step for non-hydrolyzed collagen was further evaluated. Each condition was tested in triplicate.

For both batch experiments, eight mL of faecal slurry were added to sterile serum bottles (120 mL capacity) each of which contained 32 mL of a specific nutritional medium. The bottles were then capped with butyl-rubber stoppers, flushed with N_2 and incubated for 24h at 38.5°C in a shaking incubator. At the beginning (0h; T0) and the end of incubation (24h; T24), total gas production and pH were measured. Liquid samples were collected at T0 and T24, and frozen at -20°C for subsequent analyses.

Adaptation of a long-term dynamic human gut model to a model of the cheetah GIT

The setup of the dynamic model was adapted from the SHIME (registered trademark, Ghent University and ProDigest, Belgium), a five stage sequential reactor system that simulates the different parts of the human GIT under controlled conditions as first described in 1993 by Molly *et al.* [236] and later further validated [241,446]. For this study, the specific parameters and reactor setup were changed to resemble *in vivo* conditions of the cheetah's GIT. If specific physiological data were not available for the cheetah, data from the domestic cat as closest phylogenetic relative were applied. To this end, pH, temperature, feed residence time and feed composition were changed as well as the reactor setup. The latter included no compartmentalisation of the colon due to its short length *in vivo* (± 35 cm in total in domestic cats; colon ascendens ± 2 cm; colon transversum ± 7 cm) and fast digesta transit time compared to humans [127,447], which compromises accurate estimation of residence time in colon compartments separately. Although known to be highly variable depending on diet and individual, the large intestinal transit time of domestic cats is reported to be around 12h [448]. From our field data recorded during faecal samples collections over a 3-year period, we estimated a gut transit time between 16h and 20h for captive cheetahs (Becker *et al.*, unpublished data). In the *in vitro* gut model the gut retention time for a total reactor unit (*i.e.* stomach, small intestine and colon vessel) was set at 16.5h, which is significantly shorter than the overall residence time (*i.e.* 84h) in the (human) SHIME [446]. The dynamic *in vitro* model included nine parallel units (*i.e.* stomach + small intestine + colon) to simultaneously evaluate in triplicate two substrate supplementations alongside a control (**FIG 8.1**). A single vessel was used to simulate the conditions of stomach and small intestine in a sequential batch setup, with peristaltic pumps adding twice a day a defined amount of liquid feed and pancreatic juice and emptying the vessel after 2h. To increase the number of replicates for the three intended fermentation conditions, nine continuously stirred vessels with constant volume and pH control simulated nine colons in parallel, thus making three colon vessels available for each fermentation condition. Adapted from pH measurements in various segments of the GIT in cats [449], pH range of the colon vessels was set at 5.8 to 6.1. All double-jacketed vessels were kept anaerobic by flushing with N_2 and were thermostatically controlled at the body temperature of cheetahs, *i.e.* 38.5°C.

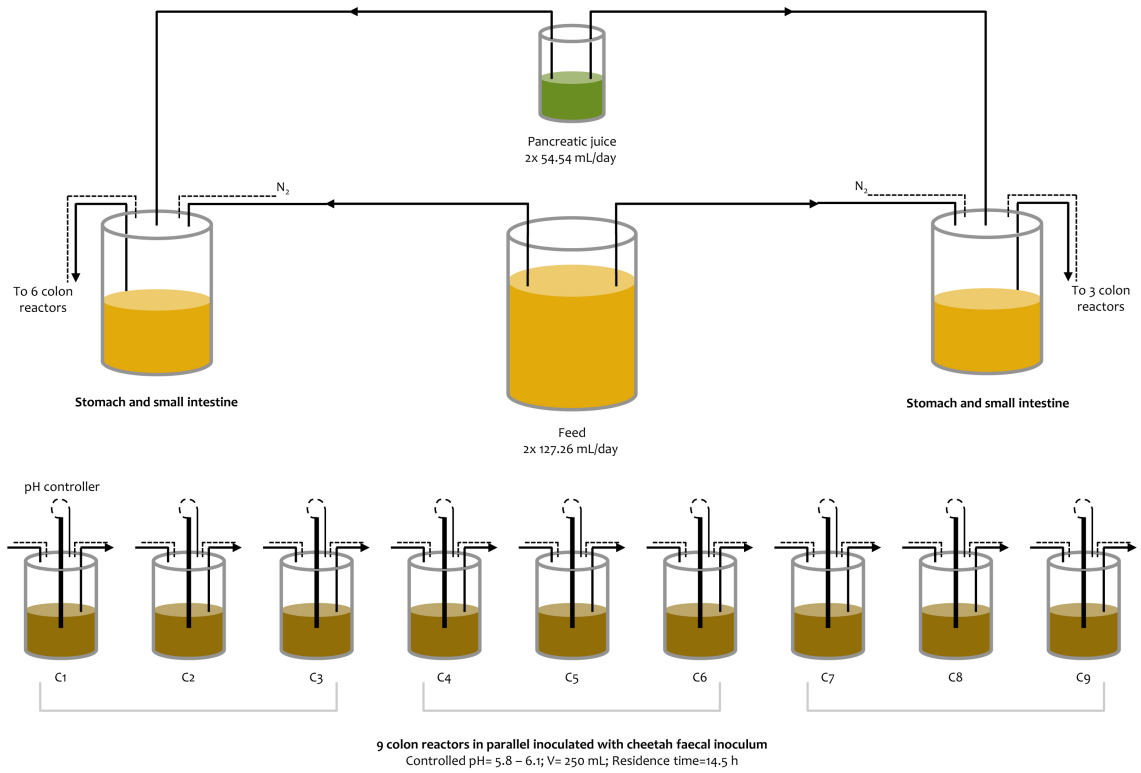


FIG 8.1 | Schematic representation of the adapted SHIME system for *in vitro* nutritional intervention studies in the cheetah's gastrointestinal tract.

Each colon vessel was inoculated with 20 mL faecal inoculum from the same cheetah NL18. The microbial inoculum was stabilized on a sugar-depleted and protein-rich feed as evaluated in batch incubations. The initial period (*i.e.* stabilization period) allowed the microbiota to adapt to the imposed *in vitro* conditions and to evolve from a faecal microbial community to one representative for the cheetah colon. Upon stabilization, a 10-day substrate supplementation period followed during which either non-hydrolyzed insoluble collagen or hydrolyzed collagen Peptan B was added to the colon vessels. Finally, test units were again solely fed the initial feed during a washout period of 6 days to determine to what extent potential substrate-induced changes were permanent upon omission of the substrate. During stabilization, supplementation and washout periods, fermentation samples were collected daily before the first feeding for metabolic activity and community composition analyses.

Metabolic analyses and profiling

Short-chain fatty acids (SCFA)

SCFA were extracted from 2 mL samples with diethyl ether after addition of 2-methyl hexanoic acid as internal standard, and measured as previously described [450]. The total SCFA production was defined as the sum of unbranched (acetate, propionate and butyrate) and branched SCFA (BCFA; isobutyrate and isovalerate). The latter were only detected above the detection limit (8-12 mg/L) in the short-term batch experiments I and II.

Ammonium

Analysis of ammonium was performed as previously described [451]. In short, a 1026 Kjeltec Auto Distillation (FOSS Benelux, Amersfoort, The Netherlands) was used to liberate ammonium in 1 mL samples by the addition of MgO as alkali compound. The released ammonia was distilled from the sample into a boric acid solution. The solution was backtitrated using a 665 Dosimat and 686 Titroprocessor (Metrohm, Berchem, Belgium).

Untargeted metabolic fingerprinting

For sample extraction, 30 µL internal standard (valine-d_8; 25 ng/ µL) was added to 1.5 mL of fermentation sample, followed by centrifugation (5 min at 13.300 x g). The supernatant was filtered through a polyvinylidene fluoride membrane (0.22 µm, 33 mm ⌀, Millex, USA) and diluted (1/5) with ultrapure water (Millipore, Brussels, Belgium) prior to injection for chromatographic separation.

An Accela UHPLC system of Thermo Fisher Scientific (San José, CA, USA) was used with an Acquity HSS T3 C18 column (1.8 µm, 150 mm x 2.1 mm, Waters) kept at 45°C for chromatographic separation of fermentation metabolites. As binary solvent system, ultrapure water (A) and acetonitrile (B) both acidified with 0.1% formic acid were used at a constant flow rate of 0.4 mL/min. A gradient profile with the following proportions (v/v) of solvent A was applied: 0-1.5 min at 98%, 1.5-7.0 min from 98% to 75%, 7.0-8.0 min from 75% to 40%, 8.0-12.0 min from 40% to 5%, 12.0-14.0 min at 5%, 14.0-14.1 min from 5 to 98%, followed by 4.0 min of re-equilibration. A 10 µL aliquot of each sample extract was injected for analysis.

Metabolic fingerprints of the batch and dynamic fermentation samples were determined by ultrahigh performance liquid chromatography coupled with a bench top Fourier Transform Orbitrap high-resolution mass spectrometry (UHPLC-Orbitrap-HRMS). The Exactive™ single-stage Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, USA) equipped with a heated electrospray ionization probe (HESI-II) operated in polarity switching mode. Ionization source working parameters were set to a sheath, auxiliary and sweep gas of 50, 25 and 5 arbitrary units (au), respectively, heater and capillary temperature of 350°C and 250°C and tube lens, skimmer, capillary and spray voltage of 60 V, 20 V, 90 V and 5 kV (+/-), respectively. A scan range of m/z 50-800 was chosen and the resolution was set at 100.000 FWHM at 1 Hz. The automatic gain control (AGC) target was set at balanced (1 x E6 ions) with a maximum injection time of 50 ms. Per data set, quality control (QC) samples prepared from a pool of all individual samples were simultaneously analyzed and used to normalize data and correct for instrumental drift. For this purpose, QC samples were dispersed evenly across the sample batch and duplicate QC injections occurred after every 10 samples. The average signal of those two injections was used for normalizing the 10 preceding samples. Samples were injected in randomized order. The protocols used for sample extraction and metabolic fingerprinting UHPLC-HRMS were previously validated [445] to ensure robust and specific data. Processing of data was performed with Xcalibur™ 2.1 (Thermo Fisher scientific, San Jose, USA). Automated peak extraction, peak alignment, deconvolution and noise removal were conducted on the obtained full scan HRMS data files for each ionization mode separately using Sieve™ 2.1 software (Thermo Fischer Scientific).

Microbial community analyses

DNA extraction and V3-16S rRNA gene PCR

The protocol of Pitcher *et al.* (1989) [315] was used in a modified version [210] to extract total bacterial DNA from fermentation samples (1 mL). DNA size and integrity were assessed on 1% agarose electrophoresis gels stained with ethidium bromide. DNA concentration and purity were determined by spectrophotometric measurements at 234, 260 and 280 nm. The variable V3 region of the 16S rRNA gene was amplified for subsequent molecular fingerprinting as previously described [401].

Denaturing Gradient Gel Electrophoresis (DGGE)

The resulting 16S rRNA gene amplicons were analyzed with DGGE fingerprinting (D-code System, Bio-Rad, Nazareth, Belgium) using a 35-70% denaturing gradient as previously described [401]. After staining with 1x SYBR Gold nucleic acid (S-11494, Invitrogen, Merelbeke, Belgium), band profiles were visualized using a charge-coupled device (CCD) camera and the Bio-Rad Quantity One software program. A standard reference lane containing the V3-16S rRNA gene amplicons of 12 taxonomically well-characterized bacterial species [354] was included every fifth lane for normalization of the fingerprint profiles using the BioNumerics software version 7.0 (Applied Maths, St-Martens Latem, Belgium). After normalization of the gels, individual bands were marked using the auto search bands option, followed by manual correction if necessary. Band intensities were calculated from the peak area in the densitometric curves. All of the profiles were compared using the band-matching tool, and uncertain bands were included in the position tolerance settings. Bands were allocated to band-classes (Bcl) which were previously defined from a collective analysis of 55 DGGE fingerprint profiles of captive cheetahs [401]. Taxonomic identity of Bcl of interest was inferred by position-based comparison with a selection of previously generated clones that together represent all phylotypes identified in two cheetahs with phylogenetic clone library analysis [351]. Bcl that could not be identified in this way were excised from the DGGE gel, eluted into 40 µL 1x TE buffer and heated for 10 min at 65°C. Subsequently, the DNA solutions were reamplified, and sequenced as previously described [401].

Data analyses

SCFA and ammonium data analysis

Statistical analyses were performed using SPSS Statistics v.22.0 software. Prior to hypothesis testing, datasets were screened for normality with Kolmogorov-Smirnov and Shapiro-Wilk tests and for homogeneity of variances with Levene's test. If so, one-way ANOVA with Scheffé's post-hoc test was performed, while otherwise a Kruskal-wallis test with subsequent Mann-Whitney U test and Bonferroni correction to investigate differences in SCFA and ammonium concentration between various conditions in the batch incubations. Statistical significance was considered at $P<0.05$. Stability of SCFA and ammonium production in the dynamic gut model was monitored by plotting Pearson correlation coefficients over time between metabolic profiles of consecutive time points.

DGGE data analysis

Taking into account both band position and band density, the Pearson correlation coefficient and unweighted-pair group method using average linkages (UPGMA) clustering algorithm were used to create dendrograms of DGGE profiles. In addition, principal component analysis (PCA) was performed to detect directional shifts within the total bacterial community. Potentially discriminating Bcl were identified by linear discriminant analysis on the DGGE datasets with BioNumerics v.7. In addition, for each experiment, quantitative information derived from relative band intensities was exported as a data matrix into SPSS v22.0 for subsequent statistical analyses.

To assess bacterial community stability in the dynamic gut model the evolution of the DGGE profile similarities between consecutive time points, *i.e.* the Pearson correlation coefficient and rate of change (100% - correlation%), was determined for each colon vessel (n=9) with moving window analysis. The latter tool was previously validated for monitoring microbial community dynamics in the SHIME reactor system [446]. For ecological interpretation of the DGGE data, two parameters were calculated from Pareto-Lorenz distribution curves *i.e.* the functional organization parameter (FO; approximate microbial community structure in terms of evenness) at the 20% level of the x- axis, and the GINI coefficient as a single value between 0 and 1 for measuring the normalized area between a given Lorenz curve and the perfect evenness line [356,408].

Metabolomics data analysis and visualization

Multivariate analysis by projection was conducted to interpret the metabolomics data with SIMCA-P+ software v.13.0. After data pre-processing (centroid scaling and log transformation), unsupervised methods (*i.e.* no prior knowledge of class membership of samples is assumed) such as PCA were used as starting point to reveal clustering patterns and outliers in data. Supervised methods were then applied to maximize the separation between classes (*i.e.* predefined groups of samples). These methods include orthogonal partial least squares with discriminant analysis (OPLS-DA), which is a prediction and regression method that finds information in the X data (*i.e.* metabolite spectra) that is related to known information, the Y data (*i.e.* predefined classes) separate from systematic variation in the data that is not correlated to Y. In this way, separation of predictive and uncorrelated (*i.e.* orthogonal) variation facilitates interpretation of metabolomics data [452]. OPLS-DA plots are visualized with Hotelling's T^2 ellipse, which defines the 95% confidence interval of the modelled variation. Model diagnostics in terms of fit (R^2X, R^2Y) and predictive ability (Q^2) were calculated to see how good the model fits the data and plots were validated by ANOVA of the cross-validated residuals (CV-ANOVA) [453]. Normalized peak intensity data were also imported into the web-based program MetaboAnalyst (http://www.metaboanalyst.ca; [454]) for further analysis, including two-way heat mapping with hierarchical clustering and pathway enrichment analysis. To filter out statistically significant - not necessarily biochemically significant – biomarkers between two groups, S-plots combined with VIP scores were used [455] next to fold change analysis with two-fold threshold and non-parametric univariate analysis using a Wilcoxon Rank test with Benjamini Hochberg's False Discovery Rate (FDR) to

correct for multiple comparisons. An adjusted $P<0.05$ (*i.e.* $q<0.05$) was considered to be statistically significant. These compounds of interest were putatively identified using a ChemSpider search within the SIEVE software and additional small-molecule and pathway databases (e.g. HMDB, KEGG, PubChem) were queried using various web tools in order to find the maximum coverage and to obtain functional information. MetaboAnalyst functional interpretation tools were used for pathway analysis and the hypergeometric test to evaluate whether a particular metabolite set is represented more than expected by chance within the given compound list. KEGG IDs and PubChem CIDs were utilized to map identified metabolites in network graphs using MetaMapp. This tool integrates Tanimoto chemical similarity matrices with biochemical mapping of single-step substrate product pairs using KEGG reactant pairs database. MetaMapp outputs are compatible with Cytoscape for visualization of network graphs [456]. Complex multivariate datasets from time series metabolic profiling of the dynamic *in vitro* gut model during substrate supplementation were analyzed additionally with ANOVA-simultaneous component analysis (ASCA) as well as evaluated for the presence of interactions between factors time and treatment [457,458].

8.3 Results

8.3.1 Testing the influence of peptone source during static short-term batch fermentations

In batch experiment I, a series of medium formulations defined by differential peptone sources were compared during 24h batch incubations with cheetah faecal inoculum. For all batch incubations, the nutritional medium was buffered and on average only slightly changed from pH 6.16 ± 0.004 to 6.23 ± 0.004 after 24h incubation. No significant relation was found between peptone source fermentation and total gas production. In all replicates, headspace pressure increased during incubation on average with 572 ± 4 hPa during incubation.

TABLE 8.2 | Concentrations of short-chain fatty acids (SCFAs) and ammonium (mmol/L; mean ± SE) after 24h incubation of cheetah faecal inoculum with four different nutritional media that vary in peptone source. Each medium condition was tested in quadruplicate.

Metabolite	M1* (10 g/L pp)	M2* (5 g/L pp + 5 g/L mp)	M3* (3 g/L pp + 7 g/L mp)	M4* (10 g/L mp)
Acetate	39.81 ± 0.40	40.23 ± 0.72	38.70 ± 0.58	40.20 ± 0.17
Proprionate	13.26 ± 0.20	13.17 ± 0.21	13.51 ± 0.16	14.57 ± 0.10^{a}
Butyrate	13.54 ± 0.26^{a}	12.18 ± 0.23^{b}	9.76 ± 0.20^{c}	8.56 ± 0.18^{d}
Isobutyrate	3.69 ± 0.02^{ab}	3.74 ± 0.04^{ab}	3.56 ± 0.06^{b}	3.76 ± 0.03^{a}
Isovalerate	6.18 ± 0.05^{a}	6.89 ± 0.03	6.71 ± 0.09	6.98 ± 0.06
Total SCFA	**76.47 ± 0.50^{a}**	**76.21 ± 1.07^{a}**	**72.25 ± 0.99^{b}**	**74.06 ± 0.25^{ab}**
Acetate/Propionate	3.0 ± 0.02^{a}	3.1 ± 0.01^{a}	2.9 ± 0.01^{b}	2.8 ± 0.03^{c}
Ammonium	80.41 ± 0.93	77.66 ± 2.73	75.08 ± 2.04	76.94 ± 2.67

***** non-variable medium components: 12.25 g/L KH_2PO_4, 1.78 g/L Na_2HPO_4, 3 g/L yeast extract, 4 g/L mucin from porcine stomach Type II

pp: proteose peptone, mp: meat peptone

Values with a different superscript indicate significant differences ($P<0.05$; ANOVA with post-hoc Scheffé tests)

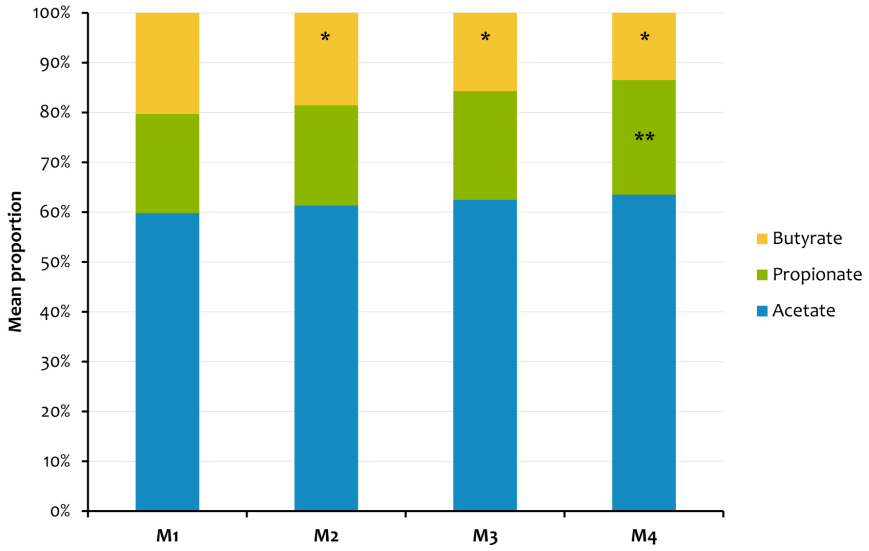


FIG 8.2 | Proportional values of SCFAs after 24h incubation of cheetah faecal inoculum in media with different peptone source combinations (M1-M4). * significant decrease compared to M1, ** significant increase compared to M1, M2, M3

The four different medium conditions (M1-M4) resulted in an average total SCFA production of 74.7 ± 0.99 mM, with low variation (max. 1 mM) between replicates (**TABLE 8.2**). In contrast to media containing proteose peptone, M4 that only contained meat peptone resulted in significantly higher propionate levels ($P<0.05$). With the gradual increase of meat peptone and decrease of proteose peptone amount in the medium, a significant decrease of butyrate as well as a significant decrease in acetate/propionate ratio were observed when comparing M1 to M4 ($P<0.05$) (**FIG 8.2**). Proportionally, an increase in meat peptone specifically stimulated propionate, whereas an increase in proteose peptone resulted in higher butyrate levels. As for the BCFA, isovalerate significantly increased when meat peptone was a component of the medium whereas isobutyrate levels were not different between media. For ammonium production, no significant differences were observed and all four different medium conditions averaged 77.5 ± 1.11 mM of ammonium production. In addition to the observed changes in SCFA ratios, the choice of peptone source also altered the microbial community composition. This was evidenced by cluster analysis and PCA of DGGE fingerprints of the total bacterial community. While DGGE fingerprints of samples with different peptone compositions initially clustered together (0h), both groups containing only one type of peptone (M1 and M4) clustered separately after 24h of incubation. DGGE fingerprints of microbial communities displayed 86.3% similarity between replicates of M4 up to 93.4% similarity between replicates of M1 (**FIG 8.3**). Independent of peptone source, linear discriminant analysis and statistical analysis of Bcl intensities identified a significant decrease after 24h incubation in band density and presence of Bcl 64.99, assigned to *Clostridium* cluster I and Bcl 50.60, assigned to *Clostridium* cluster XIVa ($P<0.05$). These taxonomic identities were inferred by position-based comparison with a selection of previously generated clones. On the contrary, Bcl 57.31 affiliated with Coprococcus comes ATCC 27758^T (*Clostridium* cluster XIVa) was significantly enriched in samples from M3 and M4 compared to M2 and M1 after 24h incubation ($P<0.05$).

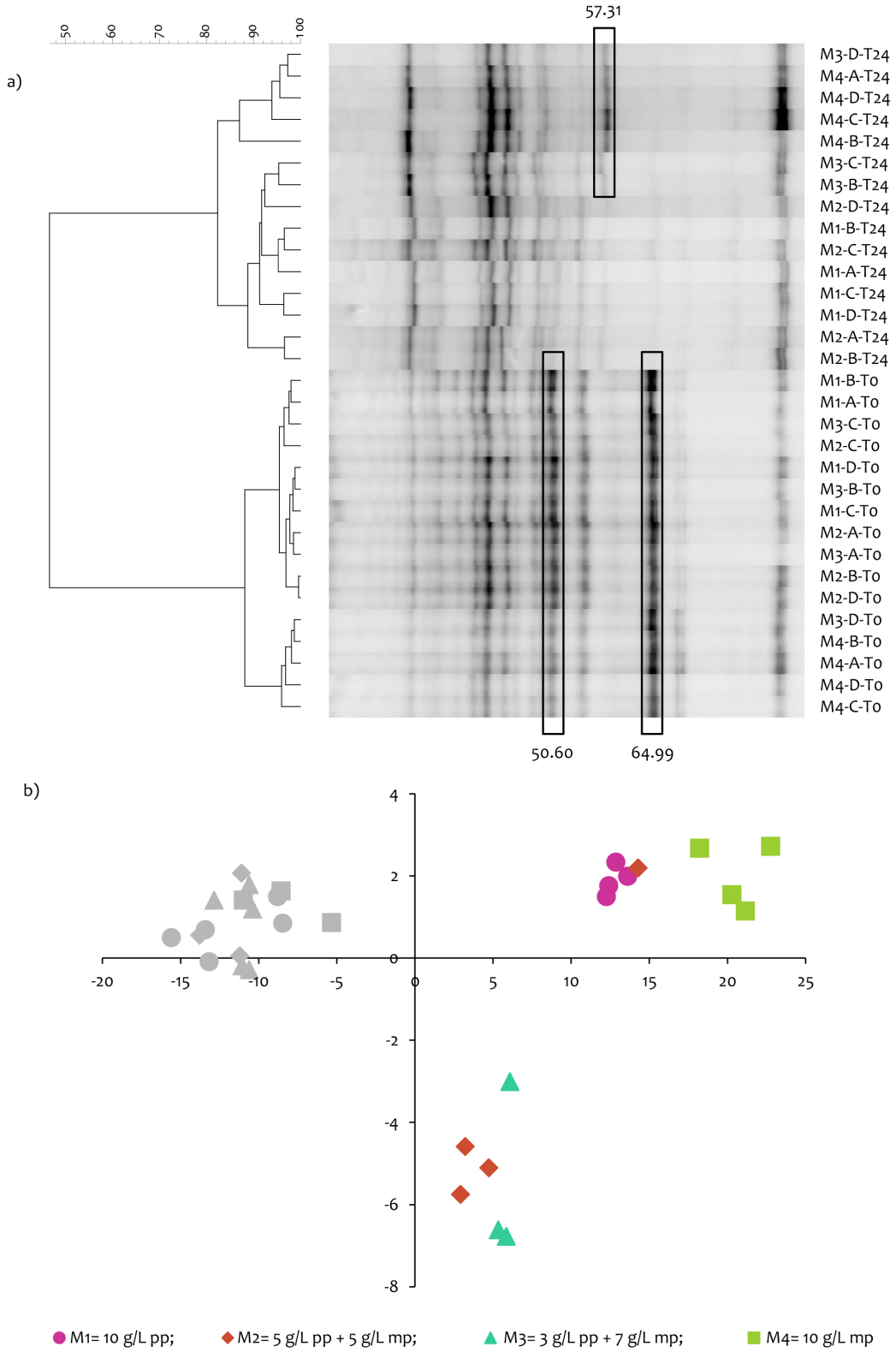


FIG 8.3 | Cluster analysis of DGGE fingerprints based on the Pearson correlation coefficient (a), and PCA of the total bacterial community (b) in fermentation samples of batch experiment I (24h incubation with cheetah faecal inoculum). Each medium condition (M1-M4) was tested in quadruplicate (A-D) with samples taken at 0h (T0; grey) and 24h (T24; colored)
pp = proteose peptone, mp = meat peptone

Metabolite fingerprints of samples from t=0 and t=24h appeared well separated in a PCA plot (**FIG S8.1**). Moreover, after 24h, 48% of the variation in the metabolic profile was related to differences in peptone source as shown in a cross-validated OPLS-DA scores plot. Replicates for each condition also clustered closely together (**FIG 8.4**). Hierarchical clustering and heat map of peak intensities depicts divergent metabolite fingerprints depending on peptone source with cluster 1 being more abundant in M4 and cluster 2 appearing to be more abundant in the M1 group (**FIG S8.2**). Non-parametric univariate analysis using FDR of 5% revealed a total of 711 metabolites that differed significantly for the negative ion mode between the M1 and M4 groups and this number was further reduced to 257 after fold-change analysis. Similarly, 1065 metabolites were significantly different for the positive ion mode (data not shown). Metabolite peak intensities for the majority of significantly differing compounds were higher after incubation of nutritional medium M4 compared to M1 (i.e. 66.2% in positive ion mode data and 63.4% in negative ion mode data). Creation of a network using only metabolites identified in the present study revealed a group of metabolites that clustered around glutamic acid (**FIG S8.3a**), and have roles in different biochemical pathways related to protein metabolism (**FIG S8.3b**).

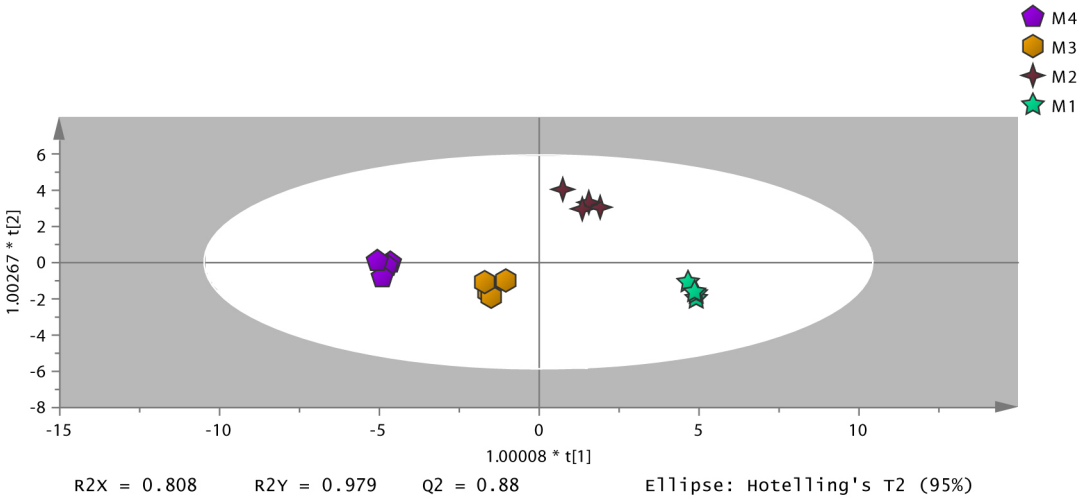


FIG 8.4 | OPLS-DA scores plot showing the divergence in metabolomic profiles after 24h incubation of cheetah faecal inoculum. Four different nutritional media (M1-M4) were tested in quadruplicate and samples were taken at 0h (T0; grey) and 24h (T24; colored).

8.3.2 Testing the influence of non-hydrolyzed and hydrolyzed collagen type I during static short-term batch fermentations

In batch experiment II, non-hydrolyzed collagen type I and hydrolyzed collagen Peptan B were compared as substrates during 24h incubation with cheetah faecal inoculum. In general, Peptan B supplementation significantly increased production of all SCFA, BCFA and ammonium compared to non-hydrolyzed collagen supplementation and control (*i.e.* meat peptone medium without substrate), whereas non-hydrolyzed collagen supplementation decreased SCFA production compared to the other test conditions ($P<0.05$) (**TABLE 8.3**). However, the acetate/propionate ratio was significantly higher compared to the control, but not different from the tested supplementation conditions (**FIG 8.5**). In

addition, higher amounts of Peptan B significantly increased propionate and butyrate as well as ammonium levels. No other significant differences were observed upon supplementation with different amounts of substrate.

TABLE 8.3 | Concentrations of short-chain fatty acids (SCFAs) and ammonium (mmol/L; mean ± SE) upon addition of 0.5 g or 1 g non-hydrolyzed collagen Type 1 or hydrolyzed collagen Type 1 (Peptan B collagen peptides) after 24h incubation of cheetah faecal inoculum. Each medium condition was tested in triplicate.

Metabolite	M1* (10 g/L pp)	M2* (5 g/L pp + 5 g/L mp)	M3* (3 g/L pp + 7 g/L mp)	M4* (10 g/L mp)
Acetate	39.81 ± 0.40	40.23 ± 0.72	38.70 ± 0.58	40.20 ± 0.17
Proprionate	13.26 ± 0.20	13.17 ± 0.21	13.51 ± 0.16	14.57 ± 0.10^{a}
Butyrate	13.54 ± 0.26^{a}	12.18 ± 0.23^{b}	9.76 ± 0.20^{c}	8.56 ± 0.18^{d}
Isobutyrate	3.69 ± 0.02^{ab}	3.74 ± 0.04^{ab}	3.56 ± 0.06^{b}	3.76 ± 0.03^{a}
Isovalerate	6.18 ± 0.05^{a}	6.89 ± 0.03	6.71 ± 0.09	6.98 ± 0.06
Total SCFA	**76.47 ± 0.50^{a}**	**76.21 ± 1.07^{a}**	**72.25 ± 0.99^{b}**	**74.06 ± 0.25^{ab}**
Acetate/Propionate	3.0 ± 0.02^{a}	3.1 ± 0.01^{a}	2.9 ± 0.01^{b}	2.8 ± 0.03^{c}
Ammonium	80.41 ± 0.93	77.66 ± 2.73	75.08 ± 2.04	76.94 ± 2.67

* non-variable medium components: 10g/L meat peptone, 12.25 g/L KH_2PO_4, 1.78 g/L Na_2HPO_4, 3 g/L yeast extract, 4 g/L mucin from porcine stomach Type II
Values with a different superscript indicate significant differences (P<0.05; ANOVA with post-hoc Scheffé tests)

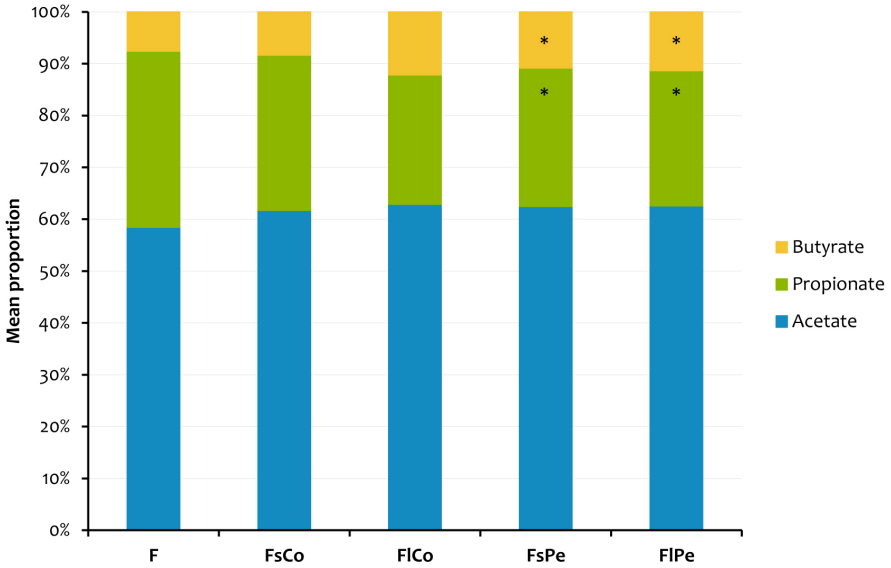


FIG 8.5 | Proportional values of SCFAs after 24h incubation of cheetah faecal inoculum with two different substrates in varying amounts. F = 10 g/L meat peptone, FsCo = F + 0.5 g collagen, FlCo = F + 1 g collagen, FsPe = F + 0.5 g Peptan B, FlPe = F + 1 g Peptan B; * indicates the significant increase of metabolites upon Peptan B supplementation compared to F and FsCo and FlCo

Hierarchical UPGMA clustering of DGGE profiles using the Pearson coefficient revealed substrate-specific clustering and statistical analysis (Kruskal-Wallis Test, P<0.05) of Bcl intensities identified five significantly discriminating Bcl (**FIG 8.6**). Bcl 38.50 (assigned to *Lactobacillaceae*), Bcl 41.28 and especially Bcl 80.93 (assigned to *Clostridium* cluster XI) showed higher intensities with Peptan B supplementation, whereas Bcl 54.43 (assigned to *Clostridium* cluster XIVa) was underrepresented upon both types of substrate supplementation compared to the control feed. Bcl 63.89 (assigned to *Bacteroides* spp.) showed significant different intensities between control and non-hydrolyzed collagen supplementation.

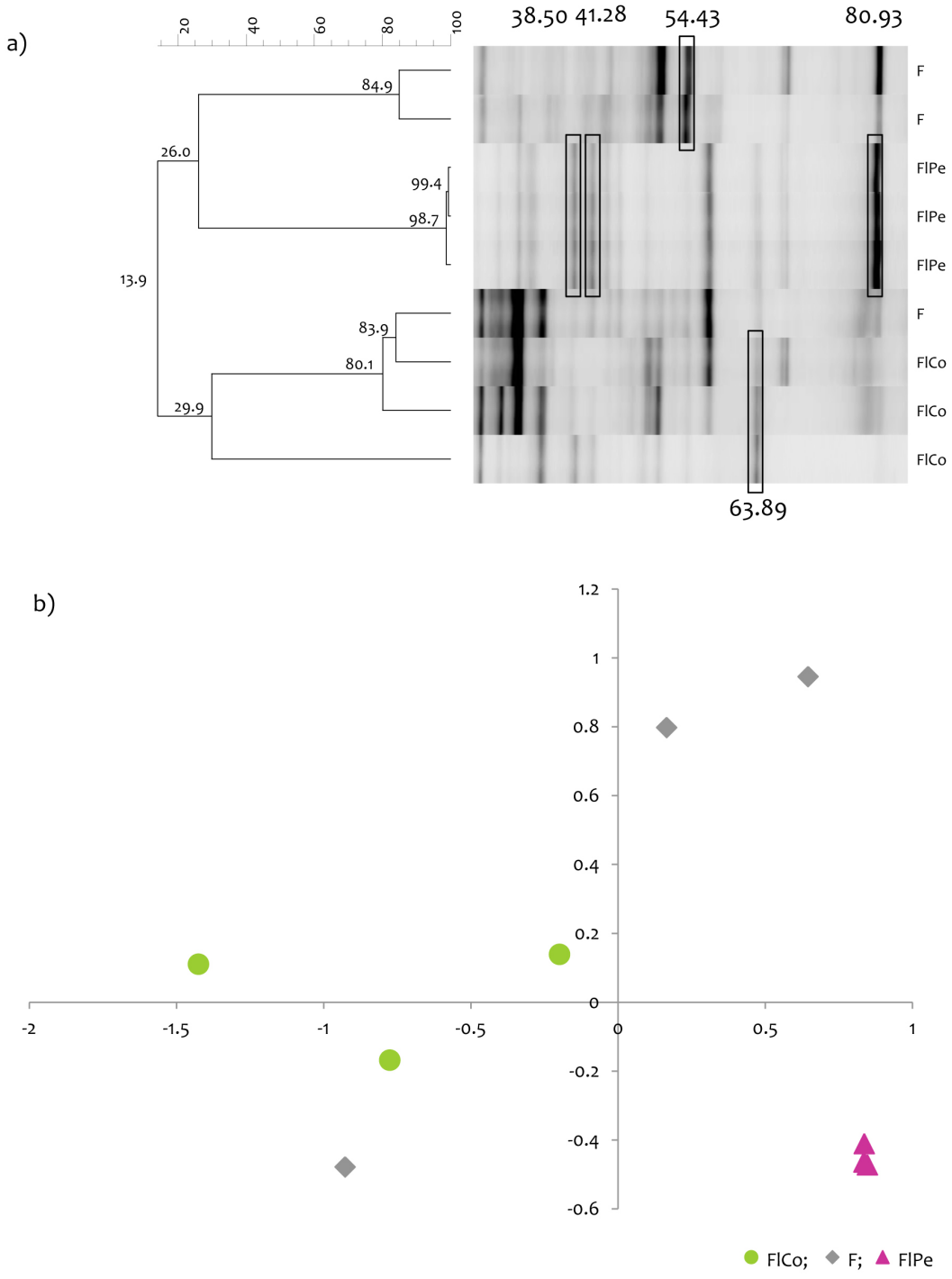


FIG 8.6 | Cluster analysis of DGGE fingerprints based on the Pearson correlation coefficient (a), and PCA of the total bacterial community (b) in samples taken after 24h incubation of cheetah faecal inoculum. Each medium condition was tested in triplicate. F= control feed *i.e.* meat peptone, FlPe = F supplemented with 1g/L Peptan B, FlCo = F supplemented with 1g non-hydrolyzed collagen

The OPLS-DA model depicts the impact of substrate type on resulting metabolite fingerprints and samples clustered separately from the control (**FIG 8.7**). The latter clustered closely with the samples obtained from a comparable medium condition (M4) in batch experiment I, which underpins the metabolite fingerprint generated solely by the nutritional medium conditions without additional substrates. Hierarchical clustering and heat mapping reveals differential metabolite fingerprints displaying various compounds with increased peak intensities upon supplementation with Peptan B (**FIG S8.4**). Non-parametric univariate analysis using FDR of 5% and fold-change analysis revealed a total of 2588 metabolites for the positive ion mode data that differed significantly between control feed and Peptan B supplementation (data not shown). Some compounds with increased concentrations in samples from the Peptan B supplemented group were identified and mapped onto the metabolic network created from batch experiment I (**FIG S8.5**).

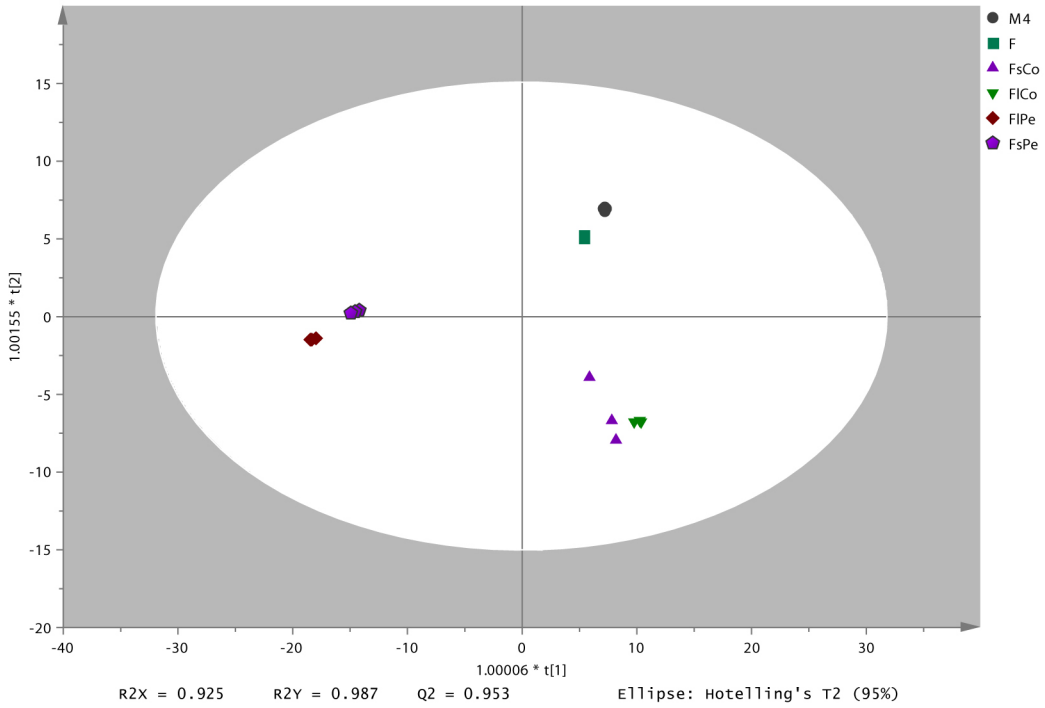


FIG 8.7 | OPLS-DA scores plot showing the divergence in metabolomic profiles after 24h incubation of cheetah faecal inoculum supplemented with (non)-hydrolyzed collagen. Supplementation of non-hydrolyzed collagen type (FsCo = + 0.5g collagen; FlCo = +1 g collagen) or hydrolyzed collagen (FsPe = + 0.5g Peptan B, FlPe = +1 g Peptan B) was tested in triplicate against the control feed (F) from batch experiment II and M4 from batch experiment I.

8.3.3 Testing the influence of enzymatic predigestion of non-hydrolyzed collagen type I during static short-term batch fermentations

In this fermentation setup, two-hour incubation with pancreatic juice preceded 24h incubation with non-hydrolyzed collagen type I. Significant increases in levels of acetate, propionate, butyrate and total SCFA were observed upon collagen predigestion (**TABLE 8.4**). DGGE fingerprints of microbial communities displayed 94.9% and 97.7% similarity between replicates of samples with predigested and non-hydrolyzed collagen, respectively. Linear discriminant analysis and statistical analysis of Bcl intensities

identified 8 discriminating Bcl between both clusters ($P<0.05$). Intensities of 4 Bcl assigned to *Carnobacteriaceae*, *Peptococcaceae*, *Enterococcaceae* and *Clostridium* cluster XI increased, whereas Bcl 50.12, affiliated with *Clostridium* cluster XIVa decreased after fermentation of digested collagen (**FIG S8.6**).

TABLE 8.4 | Concentrations of short-chain fatty acids (SCFAs) and ammonium (mmol/L; mean ± SE) upon addition of 0.5 g or 1 g non-hydrolyzed collagen Type 1 or predigested collagen Type I after 24h incubation of cheetah faecal inoculum. Each medium condition was tested in triplicate.

Metabolite	**FsCo*** **(+ 0.5 g collagen)**	**FlCo*** **(+ 1 g collagen)**	**FsCop*** **(+ 0.5 g predigested collagen)**	**FlCop*** **(+ 1 g predigested collagen)**
Acetate	5.21 ± 0.22^{a}	3.59 ± 0.30^{a}	15.80 ± 2.00^{b}	13.65 ± 0.83^{b}
Proprionate	2.53 ± 0.00^{a}	1.44 ± 0.21^{a}	11.80 ± 0.67^{b}	10.73 ± 0.04^{b}
Butyrate	0.70 ± 0.01^{a}	0.69 ± 0.03^{a}	3.56 ± 0.94^{b}	4.20 ± 0.16^{b}
Isobutyrate	0.17 ± 0.01	0.18 ± 0.02	1.13 ± 0.47	1.50 ± 0.03
Isovalerate	0.32 ± 0.01	0.32 ± 0.03	1.97 ± 0.82	2.60 ± 0.09
Total SCFA	**8.92 ± 0.23^{a}**	**6.22 ± 0.60^{a}**	**34.28 ± 4.89^{b}**	**32.67 ± 0.87^{b}**
Acetate/Propionate	2.07 ± 0.10^{a}	2.52 ± 0.13^{a}	1.33 ± 0.11^{b}	1.27 ± 0.09^{b}
Ammonium	7.96 ± 1.54	3.92 ± 0.50	81.6 ± 54.75	32.10 ± 1.61

***** non-variable medium components: 10g/L meat peptone, 12.25 g/L KH_2PO_4, 1.78 g/L Na_2HPO_4, 3 g/L yeast extract, 4 g/L mucin from porcine stomach Type II
Values with a different superscript indicate significant differences ($P<0.05$; ANOVA with post-hoc Scheffé tests)

PCA of metabolite fingerprints confirms clustering according to substrate pretreatment (**FIG S8.7**) and concentration of different metabolites increased when collagen substrate was predigested prior to incubation (**FIG S8.8**). Among metabolites with different concentrations in conditioned media, hydroxyproline, a biomarker of collagen degradation [459], was increased after predigestion (**FIG 8.8**).

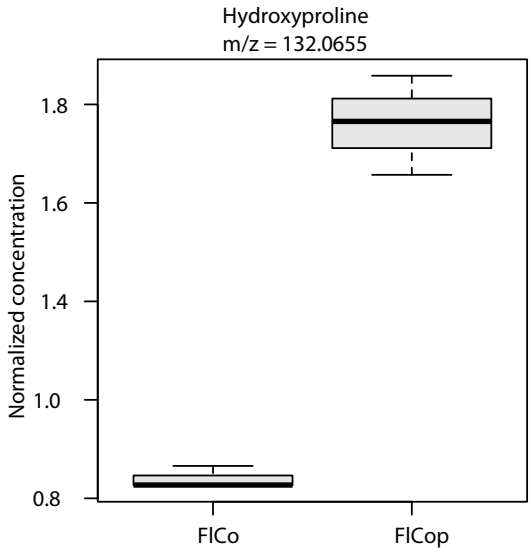


FIG 8.8 | Normalized mean values in FlCo (non-hydrolyzed collagen supplementation) and FlCop (predigested collagen supplementation) of three replicates plotted for the collagen degradation marker hydroxyproline

8.3.4 Adaptation of the human SHIME model to a cheetah GIT environment

Acetate, propionate, butyrate and ammonium levels were highly variable in all nine colon-simulating vessels during the first days after inoculation, reaching a steady-state level after approximately 14 to 18 days (**FIG 8.9**). This steady-state was characterized by less than 10% change between the metabolite profiles (acetate, propionate and butyrate) of consecutive time points for at least 5 days (**FIG S8.9**). This observation indicated that the adapted model had reached a steady-state level in terms of SCFA production for all nine reactors.

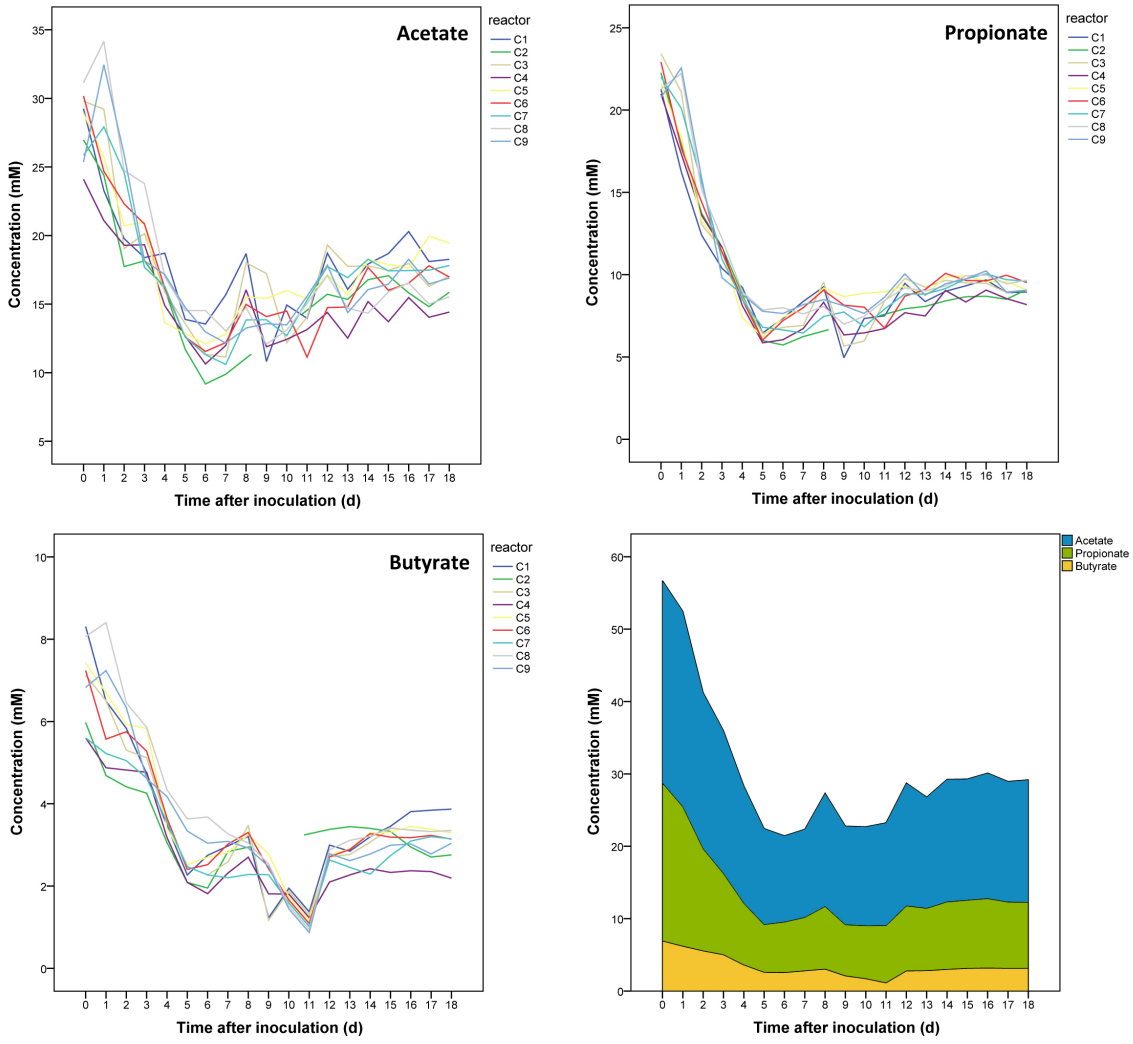


FIG 8.9 | SCFA production per colon vessel (A-C) in an adapted SHIME model simulating the cheetah GIT and the average acetate/propionate/butyrate ratio of all nine vessels depicted over the stabilization period, expressed in days (D). Data for reactor C2 are omitted for T8-T11 due to technical problems with pH controllers.

After inoculation with the cheetah faecal inoculum, the DGGE-based microbiota composition of the developing communities of each colon reactor were more similar in samples from day 1 than in samples from later time points (**FIG S8.10**). The mean rate of change between day 1 (T0) and day 14 (T14) was around 47%. After 14 days, rates of change between consecutive time points dropped below 13% for each

vessel, indicating steady-state microbial compositions (**FIG 8.10**). The FO parameter ranged between 0.45 and 0.58 throughout the stabilization period and the Gini coefficient ranged from 0.41 to 0.52, both indicating that community evenness had established in the colon reactors (**FIG S8.11**).

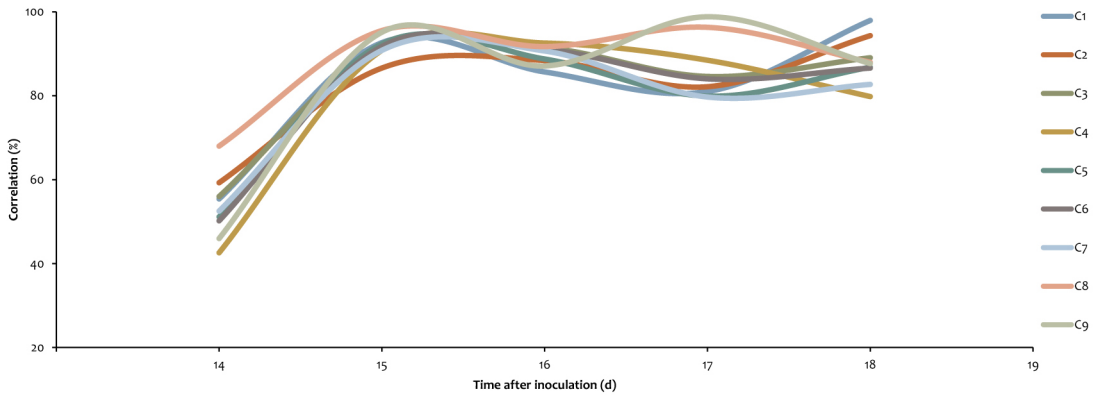


FIG 8.10 | DGGE profile similarity evolution of T0 and T14 (day 14) and consecutive time points T14-T18 (day 15 - 18) during the stabilization period after inoculation of nine colon-simulating vessels with cheetah faecal inoculum

8.3.5 Effect of (non-) hydrolyzed collagen supplementation on SCFA production and community composition in a cheetah-adapted dynamic model

After stabilization of the adapted dynamic model (day 18), supplementation with either hydrolyzed collagen (*i.e.* Peptan B) (reactors C4-C6) or non-hydrolyzed collagen (reactors C7-C9) during a 10-day period was compared to the control group (reactors C1-C3). Supplementation of vessels with Peptan B resulted in variable high levels of acetate, propionate, butyrate and ammonium compared to the control group and the group supplemented with non-hydrolyzed collagen (**FIG S8.12**), but only levels of propionate were significantly higher ($P<0.05$). For the groups supplemented with non-hydrolyzed collagen, acetate, propionate and butyrate levels even decreased compared to other test conditions during the second half of the treatment period (**FIG 8.11**). Within 24h after supplementation, levels of different SCFAs were more comparable between the groups although high variation between the vessels remained. Compared to the control group, no significant differences in acetate/propionate/butyrate ratio in the colon vessels were observed for both treatments, but slightly higher total SCFA levels resulted from supplementation with Peptan B ($P= 0.07$) (**FIG 8.12**).

Hierarchical UPGMA clustering of fingerprints did not reveal separate grouping of treatment groups. Likewise, PCA of DGGE fingerprints did not result in a separation of community profiles in function of substrate supplementation or temporal succession (**FIG S8.13**).

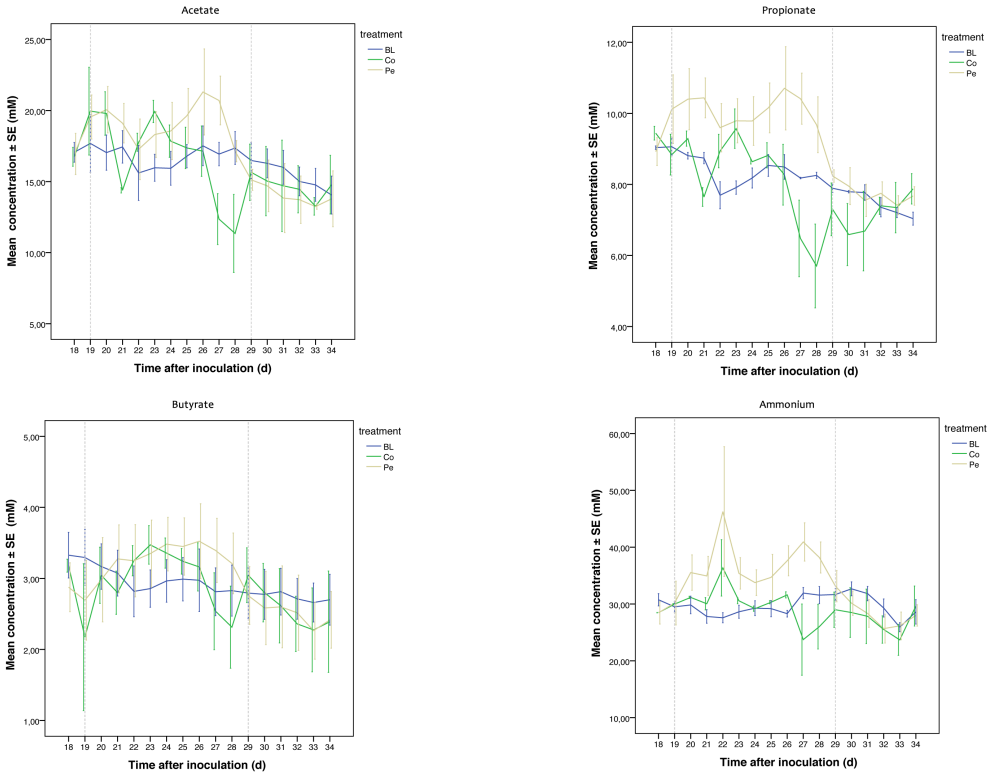


FIG 8.11 | Production of the three main SCFAs and ammonium in an adapted SHIME model simulating the feline GIT during treatment (T19-T28) and washout period (T29-T34). All conditions were tested in three parallel colon vessels. BL = control group; Pe = supplementation with hydrolyzed collagen (Peptan B); Co = supplementation with non-hydrolyzed collagen type I

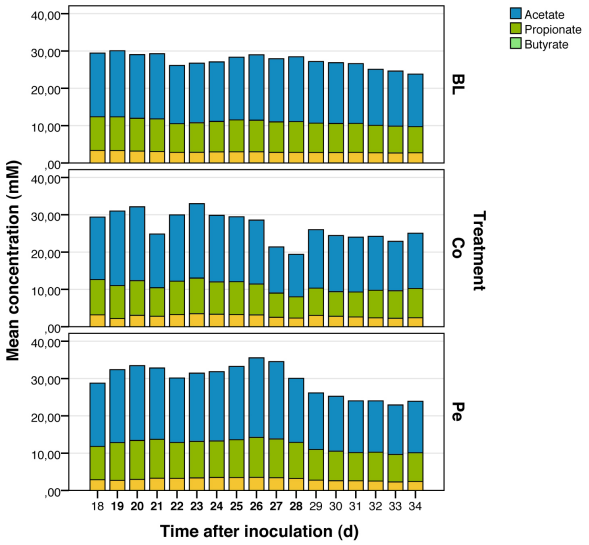


FIG 8.12 | Mean ratios of acetate, propionate and butyrate in an adapted SHIME model simulating the feline GIT during treatment (T19-T28) and washout period (T29-T34). All conditions were tested in three parallel colon reactors. BL = control group; Pe = supplementation with hydrolyzed collagen (Peptan B); Co = supplementation with non-hydrolyzed collagen type I

8.3.6 Effect of (non-) hydrolyzed collagen supplementation on metabolic fingerprints in a cheetah-adapted dynamic model

The OPLS-DA model showed significant differences in metabolite fingerprint between control group and substrate supplemented groups and the replicates for each group clustered closely together (**FIG 8.13**). Pre-processing of data reduced the positive ion mode data to a selected list of 4869 metabolites. To further identify major variations associated with each treatment condition, temporal profiles were compared across the three different conditions and possible interaction between time and treatment was investigated. A significant interaction effect was detected for 2123 metabolites, whereas 178 and 711 metabolites exhibited a significant effect of time or treatment, respectively ($P<0.05$). In addition, 45 metabolites exhibited a significant effect of both time and treatment. In contrast, 1632 metabolites were not affected by either time or treatment conditions (**FIG S8.14**). The ASCA method identified major patterns with regard to the two given factors and their interaction, as shown for example for the factor treatment in **FIG 8.14**. Important metabolites are grouped into two categories. A first subset of 249 metabolites were considered 'well-modelled', which refers to the fact that they change co-coordinately to the major profiles depicted in the trajectory plots of the factor treatment. The other category includes 'outliers', which refers to those metabolites with relevant changes but different from the major profiles.

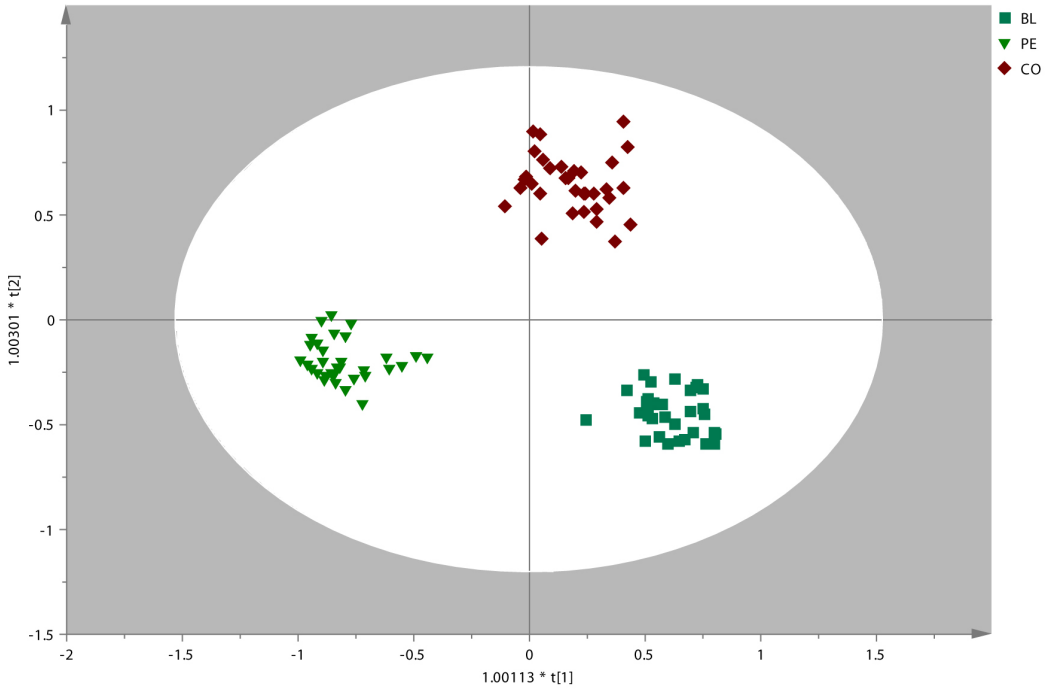


FIG 8.13 | OPLS-DA scores plot derived from UHPLC-Orbitrap-HRMS metabolomics showing the different clusters of samples following substrate supplementation. During a 10-day treatment, digestive fluids were obtained daily from a dynamic *in vitro* gut model simulating the feline GIT. Control feed (BL), feed with Peptan B (PE) and feed with non-hydrolyzed collagen supplementation (CO)

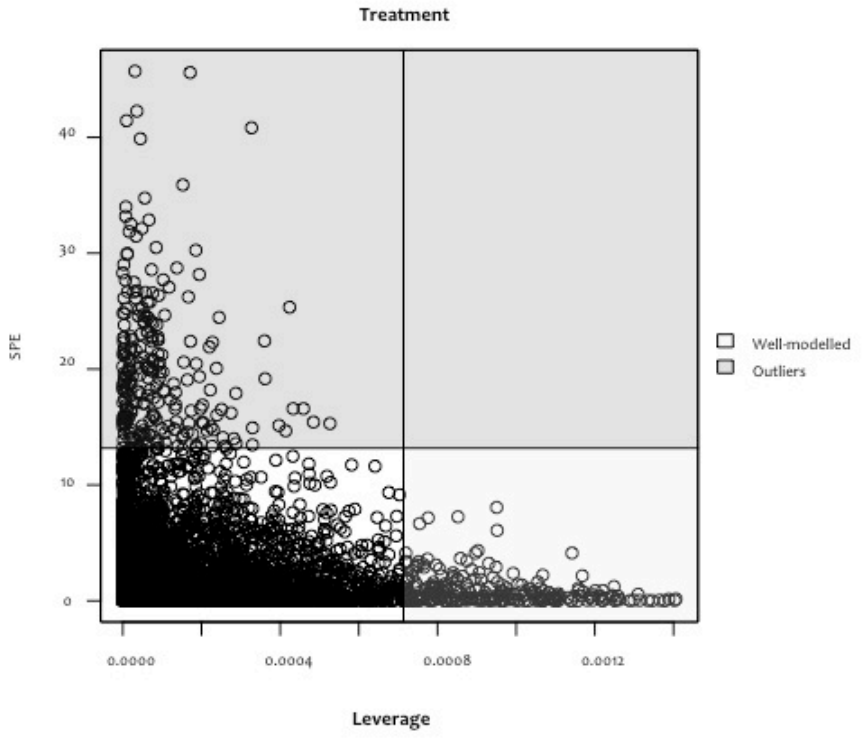


FIG 8.14 | ANOVA-simultaneous component analysis (ASCA) selection of important variables associated with treatment

8.4 Discussion

The approach used to convert a well-validated dynamic simulator of the human intestinal ecosystem (SHIME) to a model mimicking a feline GIT environment, with focus on the cheetah, comprised three major stages. In the first stage, the *in vitro* model was adapted by adjusting its fermentation parameters (*i.e.* pH, gut residence time, temperature) to mimic gastrointestinal conditions characteristic of the feline host target group and by reformulating its nutritional medium to mimic a strict carnivore's feed. The latter aspect is particularly challenging, as metabolic readouts of any experiment in *in vitro* models will be largely influenced by substrate availability. For instance, *in vitro* experiments have shown that the dissimilatory metabolism of amino acids and peptides was reduced when fermentable carbohydrates were available [460]. A feline's natural diet is typically high in animal-derived proteins and low in carbohydrates [249], for which reason the original SHIME medium was depleted of starch and polysaccharides and the amount of peptone as the main substrate source was increased. In the first static short-term batch fermentations with cheetah faecal inoculum, fermentation of peptone derived from peptic digested fresh meat resulted in lower butyrate production and higher propionate production compared to proteose peptone. The acetate:propionate:butyrate ratio resulting from fermentation of meat peptone (*i.e.* 4.6:1.7:1) was comparable to the ratio measured in colon luminal contents of domestic cats (*i.e.* 4:1.6:1) [449], in contrast to the ratio derived from fermentation of proteose peptone (i.e. 2.9:0.98:1). Moreover, the acetate:propionate ratio resulting from fermentation of meat peptone (*i.e.* 2.8 ± 0.03) was comparable to values previously measured in faecal excreta of

cheetahs fed supplemented raw beef (*i.e.* 2.9 ± 0.06) [49] or horsemeat (*i.e.* 2.8) [306]. Given the fact that animal-derived proteins as provided by meat peptone more closely resemble the *in vivo* substrate influx reaching the gut microbiota in cheetahs and result in acetate:propionate:butyrate ratios comparable to *in vivo* measurements, meat peptone was further used as the main component of a carbohydrate-depleted medium to sustain the dynamic *in vitro* gut model. The production of BCFA, exclusively derived from breakdown of the amino acids valine, isoleucine and leucine [146], and ammonium further underpin the protein fermentative capacity of the microbial community in the cheetah faecal inoculum. The predominant KEGG functions in the feline microbiome include amino acid metabolism [297] and most metabolites identified in the metabolic fingerprints derived from the fermentation samples could be mapped to pathways related to protein metabolism.

The influence of peptone source on metabolic readouts was also evidenced by OPLS-DA analysis following the untargeted metabolic fingerprinting, which revealed 257 metabolites with significantly altered peak intensity between meat peptone and proteose peptone supplementation. However, due to the paucity of database entries with specific reference to microbial metabolites, confirming the chemical identity of detected *m/z* values remains a major hurdle in metabolomics [452]. The fact that only 8% of the discriminating metabolites could be unambiguously identified necessitates the combined use of untargeted metabolic fingerprinting and metabolite profiling as the approach to study diet-induced alterations in preliminary batch fermentation experiments. In addition, the possible contribution of unfermented medium compounds other than those produced by fermentation to the metabolite fingerprints needs to be further investigated by comparing metabolomic fingerprints from media extracts and fermentation samples.

PCA of microbial community profiles showed that the peptone source had a directional effect within the total bacterial community by grouping all samples per medium condition together. An increase in density of a Bcl corresponding to *Coprococcus* spp. was found in meat peptone media, which may contribute to the higher propionate production resulting from these specific medium conditions. Although growth performance of microorganisms can vary from one peptone to another, it should be noted that conclusions drawn here are based on faecal batch incubations which are less suitable for studying overall microbial population effects. Moreover, the effect of imposed *in vitro* conditions of short-term incubation on the faecal microbial community was stronger as evidenced by the decreased density of two Bcl corresponding to members of *Clostridium* cluster I and XIVa in all samples. However, other bands corresponding to these clusters remained present and overall, neither media resulted in a bacterial community with an aberrant profile compared to that obtained from faecal samples of cheetahs. Moreover, both media supported short-term growth of the cheetah-specific bacterial community characterized by a dominant core of *Clostridium* clusters I, XI and XIVa members.

In the second stage, the adapted dynamic model mimicking a cheetah GIT environment was evaluated for its ability to reach a steady-state in terms of metabolic activity and microbial composition. Temporal stability is an important prerequisite if an *in vitro* model is to be used for assessing the modulating

potency of dietary substrates, prebiotics, probiotics or therapeutic compounds. According to standard SHIME procedures [444], the *in vitro* model was inoculated by a single fresh faecal sample from one cheetah. Pooled inocula are not recommended here because this may result in a microbial diversity that is atypically high and may disturb cross-feeding interactions that are crucial in maintaining metabolic homeostasis [444]. Recently, pooled inocula were advocated to be suitable for use in the continuous *in vitro* model of the human colon TIM-2 as they may be more representative for the whole population [461]. Although this strategy may result in representative fermentation profiles on the short term, the pooling approach remains debatable, especially when considering representative colonization profiles and microbial adaptations to changing environmental conditions.

To determine metabolic homeostasis in a dynamic *in vitro* gut model, a stability criterion based on DGGE fingerprinting and SCFA measurements was previously established by the use of moving window correlation analysis. Upon ≥80% correlation during consecutive days, the community is generally considered to be stable while the remaining 15-20% variability is thought to originate from natural biological fluctuations [446]. Monitoring of SCFA production during reactor start-up revealed an initial peak of change, possibly due to the transition from *in vivo* to *in vitro* conditions. This was followed by a gradual progression towards a steady-state as evidenced by <10% change in metabolite profiles (SCFAs) and <13% rate of change in microbial community profiles in all colon vessels after 14 days, a period that is comparable to the one required for stabilizing the validated dynamic *in vitro* model of the human GIT [446]. In comparison, faecal sample analysis has shown that captive cheetahs host an overall stable microbial community with <25% change between consecutive samples over a 3-year period [401]. Also, the steady-state in terms of comparable metabolic and microbial profiles (SCFAs) was reproducible between the nine parallel colon units, and the GINI coefficients were also indicative of well-adapted communities with adequate species distribution [408].

In the third and final stage, non-hydrolyzed and hydrolyzed (Peptan B) collagen were used as test substrates for dietary intervention in static batch fermentations and in the stabilized dynamic model. Collagen type 1 is the most abundant fibrous and structural protein in vertebrate tissues, being present in bone, tendons, ligaments and skin and constituting a major component of the free-ranging cheetah's diet [52,462]. The hydrolyzed form, Peptan B, contains 18 amino acids, characterized by the predominance of glycine (20.6%), proline (11.5%) and hydroxyproline (11.4%), which are the basic chemical building blocks of collagen fibrils. In short-term static batch fermentations (24h), Peptan B resulted in a significant increase in propionate, butyrate, total SCFA, BCFA and ammonium compared to the non-hydrolyzed collagen and control group. These metabolic changes were mirrored by changes in the metabolome and microbial composition. Discriminative DGGE BcI analysis indicated a stimulation of members of *Clostridium* cluster XI and *Lactobacillaceae*, two core members of the faecal microbial community in captive cheetahs and many other carnivores [265,331,344,351,401] that are likely to play a role in protein fermentation under the carbohydrate-depleted conditions typically found in carnivorous mammals.

In the dynamic model, however, microbial changes were far less substrate-specific as evidenced by a lack of specific grouping among community fingerprints of samples from control, non-hydrolyzed collagen or Peptan B supplemented vessels. Whereas acetate, propionate and total SCFA production were all higher in the Peptan B group, only for propionate was this difference significant. In search of possible explanations, it was important to first verify the reproducibility and stability of the model. The overlap in metabolomic features associated with the treatment groups between replicates provided a good indication of the *in vitro* model's robustness within each test condition. Moreover, metabolite profiles (SCFAs) of the control vessels only changed gradually after 30 days, which also indicates the model's capacity to continue the steady-state for at least two more weeks after initial stabilization. This was further corroborated by microbial community clustering profiles exhibiting similarity levels between 72.0 and 82.2 %, which meets the expectations of a stable SHIME [446]. Obviously, retaining long-term stability is an absolute requirement for evaluation of fermentation kinetics of dietary compounds which are generally supplemented during 3 or more weeks [240,463].

It cannot be excluded that chemical composition, physical form and fermentable amount of some substrates may be varying during the supplementation period and thus affect bacterial fermentation reactions. In the batch experiments, non-hydrolyzed insoluble collagen fibrils appeared to be inert to fermentation and their presence in the fermentation fluid resulted in decreased SCFA production. This effect was also observed in the dynamic *in vitro* gut model, though less pronounced. As native collagen has a great tensile strength and is hard to digest, the 10-day supplementation period may have been too short to observe any effect. Enzymatic hydrolysis of collagen releases specific collagen amino acids such as hydroxyproline, and converts the substrate into a fermentable compound resulting in increased SCFA and ammonium levels after 24h incubation with cheetah faecal inoculum. Although collagen is an important component in the diet of carnivores, results from this study do not indicate that its native form can serve as a substrate for the gut microbial communities and thus act as an insoluble animal fibre as previously suggested [52]. However, results from *in vitro* fermentations suggest that pepsin can break down collagen sufficiently for it to be digested by pancreatic enzymes [464]. In addition to collagen, other dietary components such as cartilage have also been proposed to act as potential animal fibre [52] in captive cheetahs. The dynamic model adapted and evaluated in the present study is highly suitable to further study the fermentation kinetics of these other substrates, on the basis of which it could be decided whether further *in vivo* studies are worth pursuing.

8.5 Conclusion

The results of this study set the stage for the adaptation of the validated human SHIME model towards a reproducible dynamic model mimicking the cheetah GIT under carbohydrate-depleted protein-rich conditions. While the combination of SCFA analysis, microbial community fingerprinting and untargeted metabolic fingerprinting proved a powerful approach to evaluate and monitor the steady-state phase of the model, additional fine-tuning is required towards its use for *in vitro* nutritional intervention studies. Especially in the case of poorly fermentable or inert substrates, the role of predigestion in the model needs to be further addressed. Even in the absence of definitive compound annotation, untargeted metabolomics proved useful for comparative analyses between medium formulations and in function of temporal stability. However, if specific components are to be tested for their modulating properties in the model, the addition of targeted metabolomics focussing on a set of known substrate-derived metabolites may be required to reveal more specific cause-effect relationships.

SUPPLEMENTARY DATA

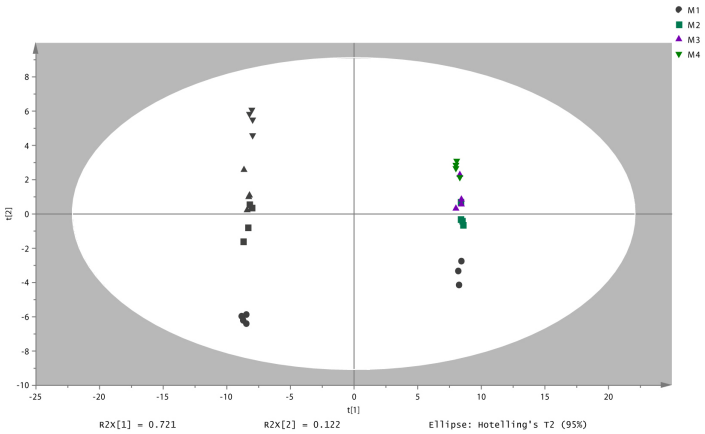


FIG S8.1 | PCA scores plot showing the divergence in metabolomic profiles after 24h incubation of cheetah faecal inoculum. Four different nutritional media (M1-M4) were tested in quadruplicate and samples were taken at 0h (T0; grey) and 24h (T24; colored).

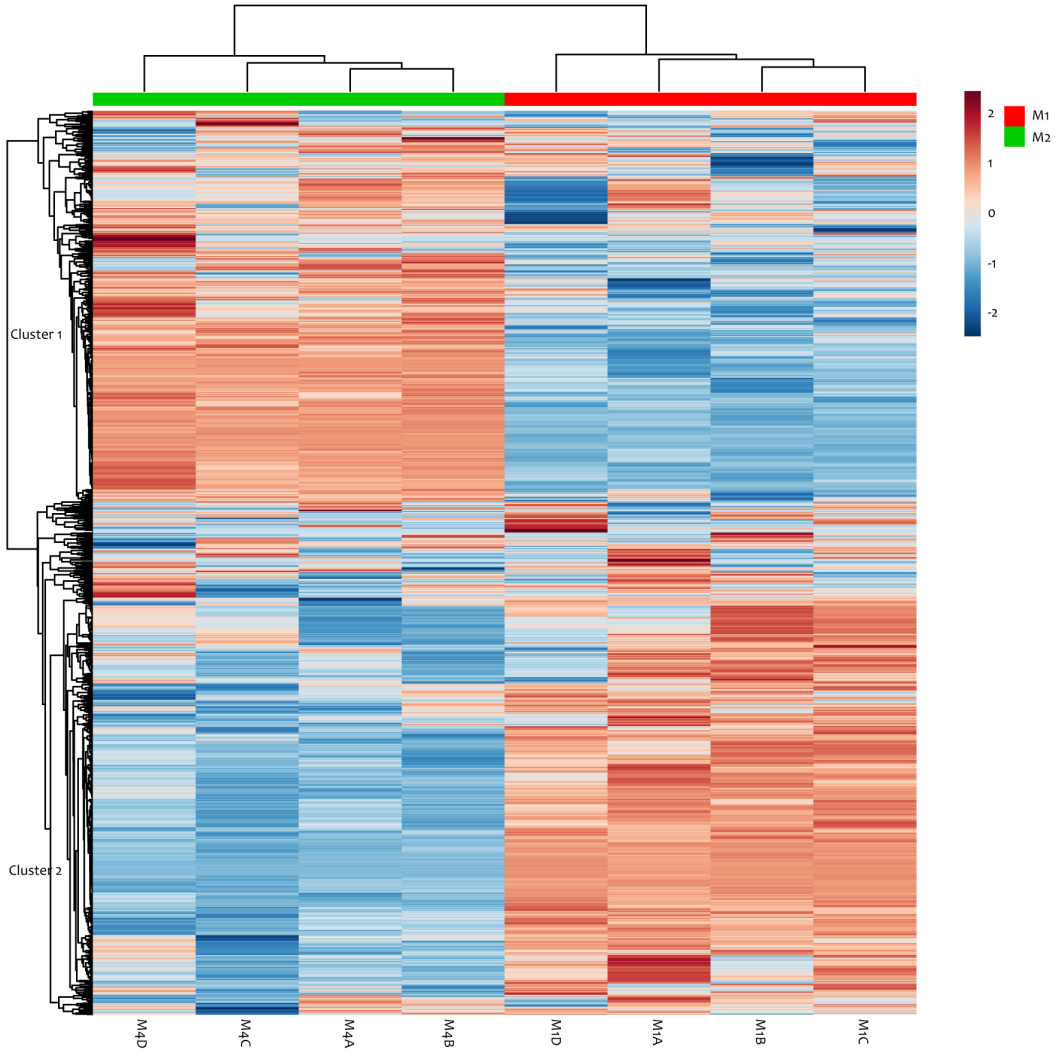
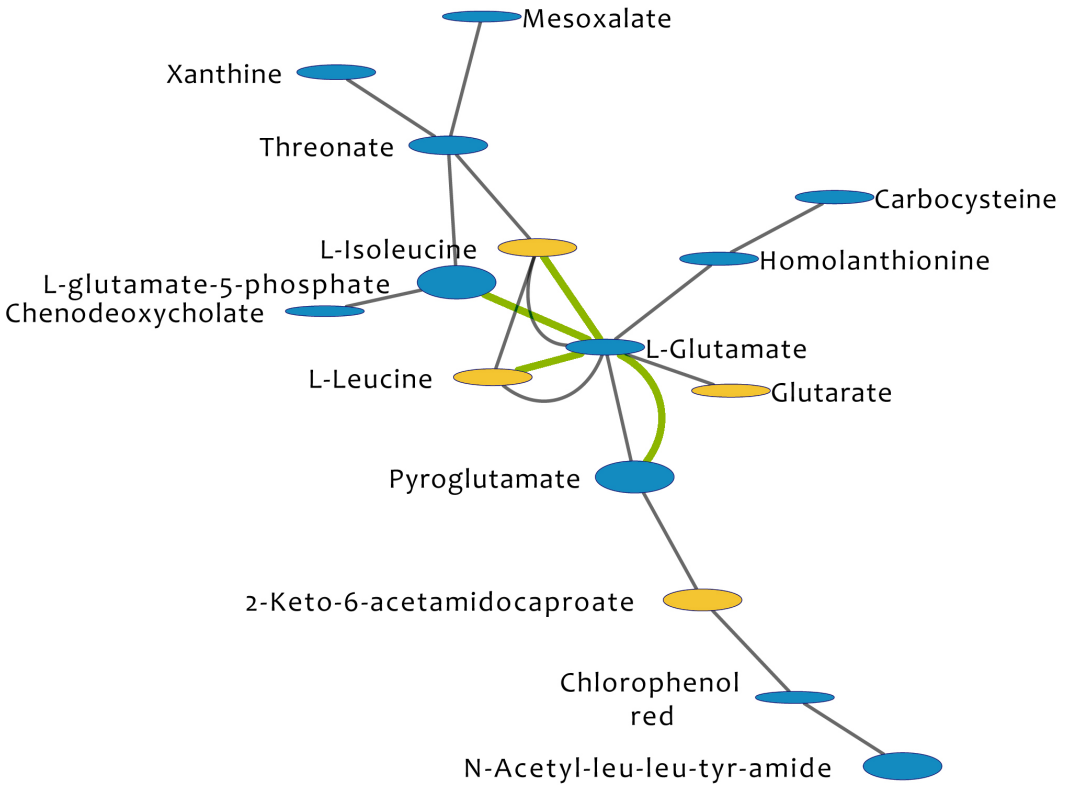


FIG S8.2 | Hierarchical clustering heatmap showing divergent metabolomic profiles after 24h incubation of cheetah faecal inoculum with different peptone sources. Nutritional media M1 (proteose peptone) and M4 (meat peptone) were tested in quadruplicate (A-D) and samples were taken after 24h.

a)



b)

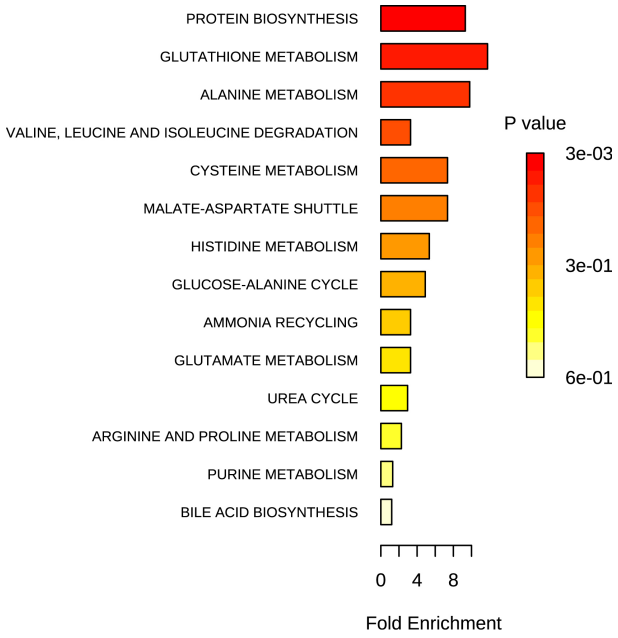


FIG S8.3 | Network analysis of identified metabolites in fermentation samples from short-term (24h) batch incubations with cheetah faecal inoculum (a). Nodes represent metabolites, green edges denote KEGG reactant pair links and grey edges symbolize structural Tanimoto chemical similarity at T > 700. Yellow nodes indicate metabolites that increase significantly in concentration in M1, blue nodes indicate metabolites that increase significantly in concentration in M4. Size of the nodes is proportional to the fold change. **Metabolic pathway analysis related to the identified metabolites that significantly differ in conditions M1 and M4 from batch experiment I, utilizing the MetaboAnalyst functional interpretation tools (b).** The horizontal bars summarize the main metabolite sets identified in this analysis; the bars are colored based on their *P*-values (Hypergeometric Test) and the length is based on the fold enrichment.

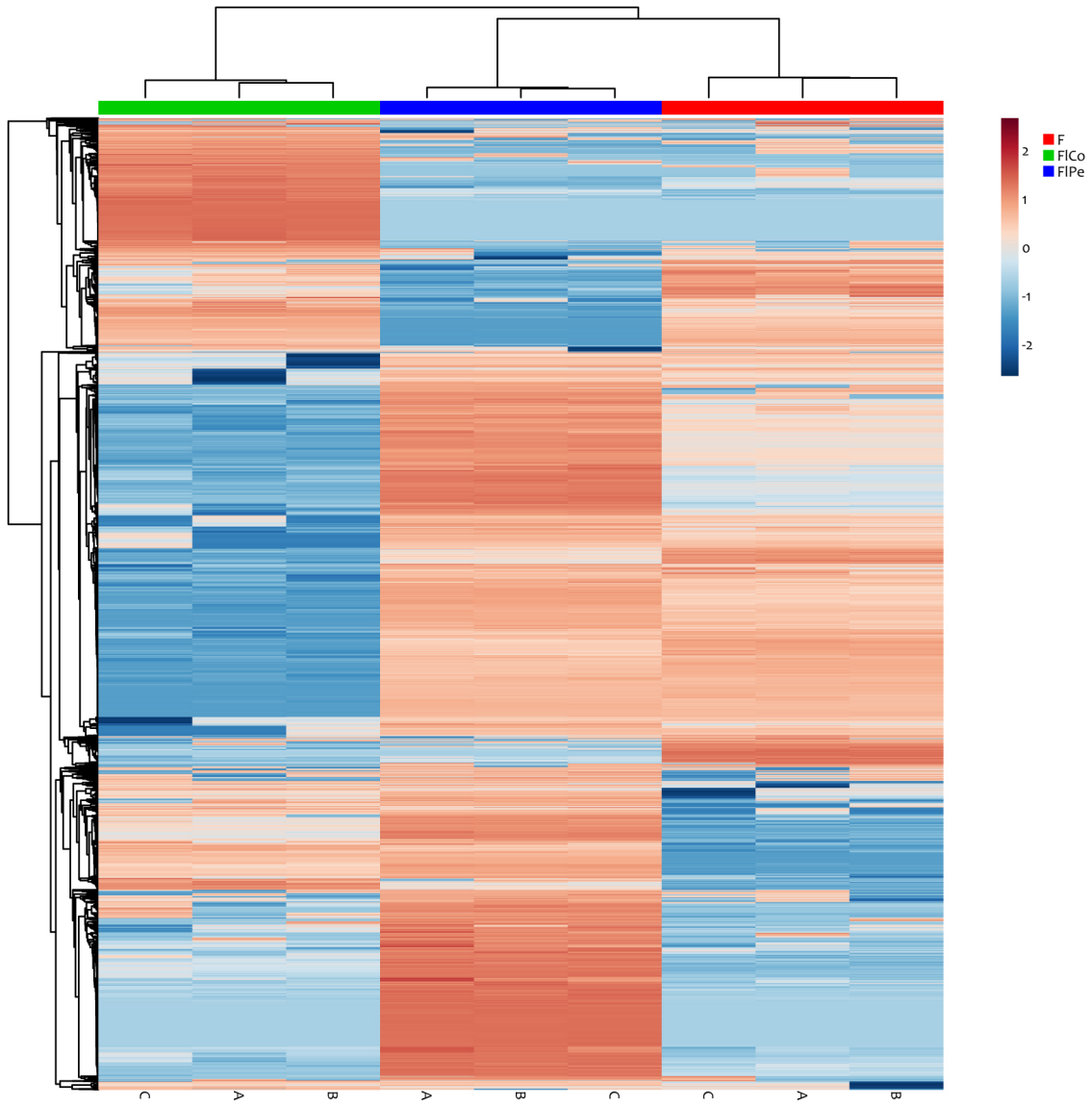


FIG S8.4 | Hierarchical clustering heatmap showing divergent metabolomic profiles after 24h incubation of cheetah faecal inoculum with (non)-hydrolyzed collagen. All medium conditions were tested in triplicate (A-C) and include the control medium F, medium FlCo (+1 g non-hydrolyzed collagen) and medium FlPe (+1 g Peptan B).

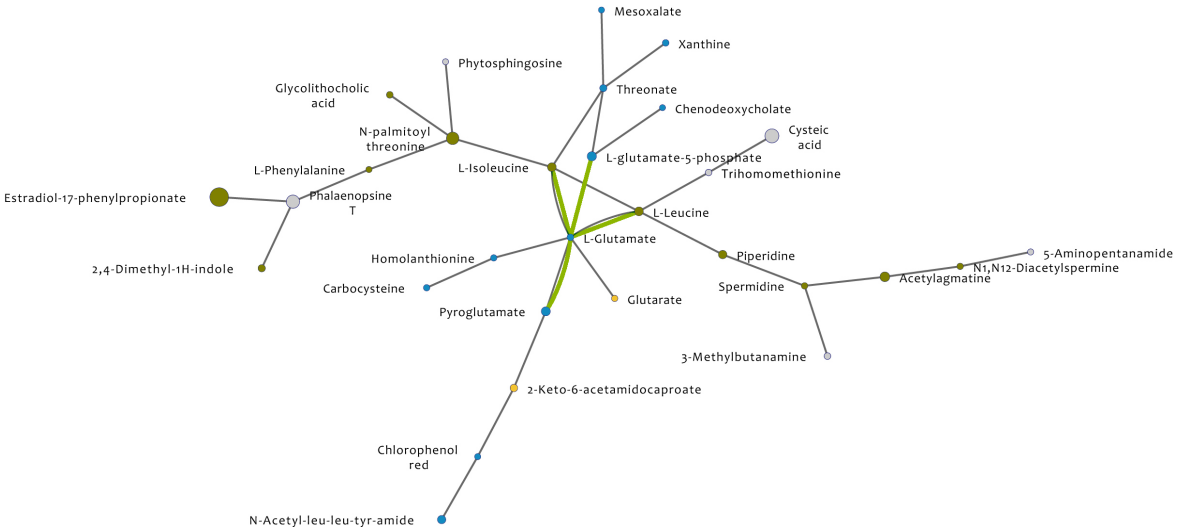


FIG S8.5 | Network analysis of identified metabolites in fermentation samples from 24h batch incubations with cheetah faecal inoculum. Nodes represent metabolites, green edges denote KEGG reactant pair links and grey edges symbolize structural Tanimoto chemical similarity at T > 700. Yellow nodes indicate metabolites that increase significantly in concentration in M1, blue nodes indicate metabolites that increase significantly in concentration in M4 (from batch experiment I). Green nodes indicate metabolites that increase significantly in concentration after Peptan B supplementation compared to the control feed F from batch experiment II, which has increased concentration of metabolites depicted with grey nodes. Size of the nodes is proportional to the fold change.

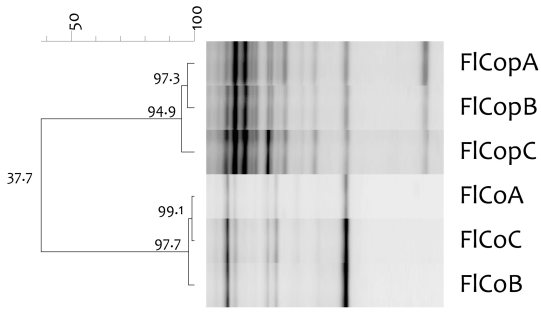


FIG S8.6 | Dendrogram based on Pearson and UPGMA correlations of the DGGE profiles obtained after 24h incubation of cheetah faecal inoculum with either non-hydrolyzed collagen (FlCo) or enzymatic predigested collagen (FlCop). All medium conditions were tested in triplicate (A-C).

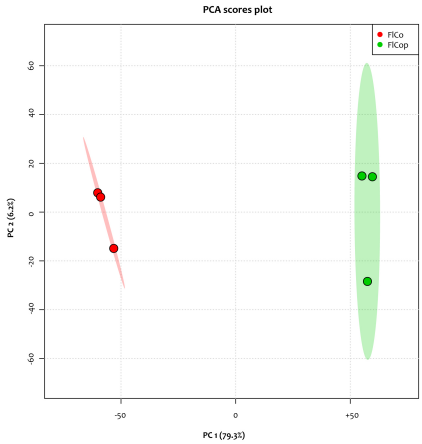


FIG S8.7 | PCA scores plot showing the divergence in metabolomic profiles after 24h incubation of cheetah faecal inoculum. Two medium conditions were tested in triplicate and include nutritional medium supplemented with 1g non-hydrolyzed collagen (FlCo) or 1g predigested collagen type I (FlCop).

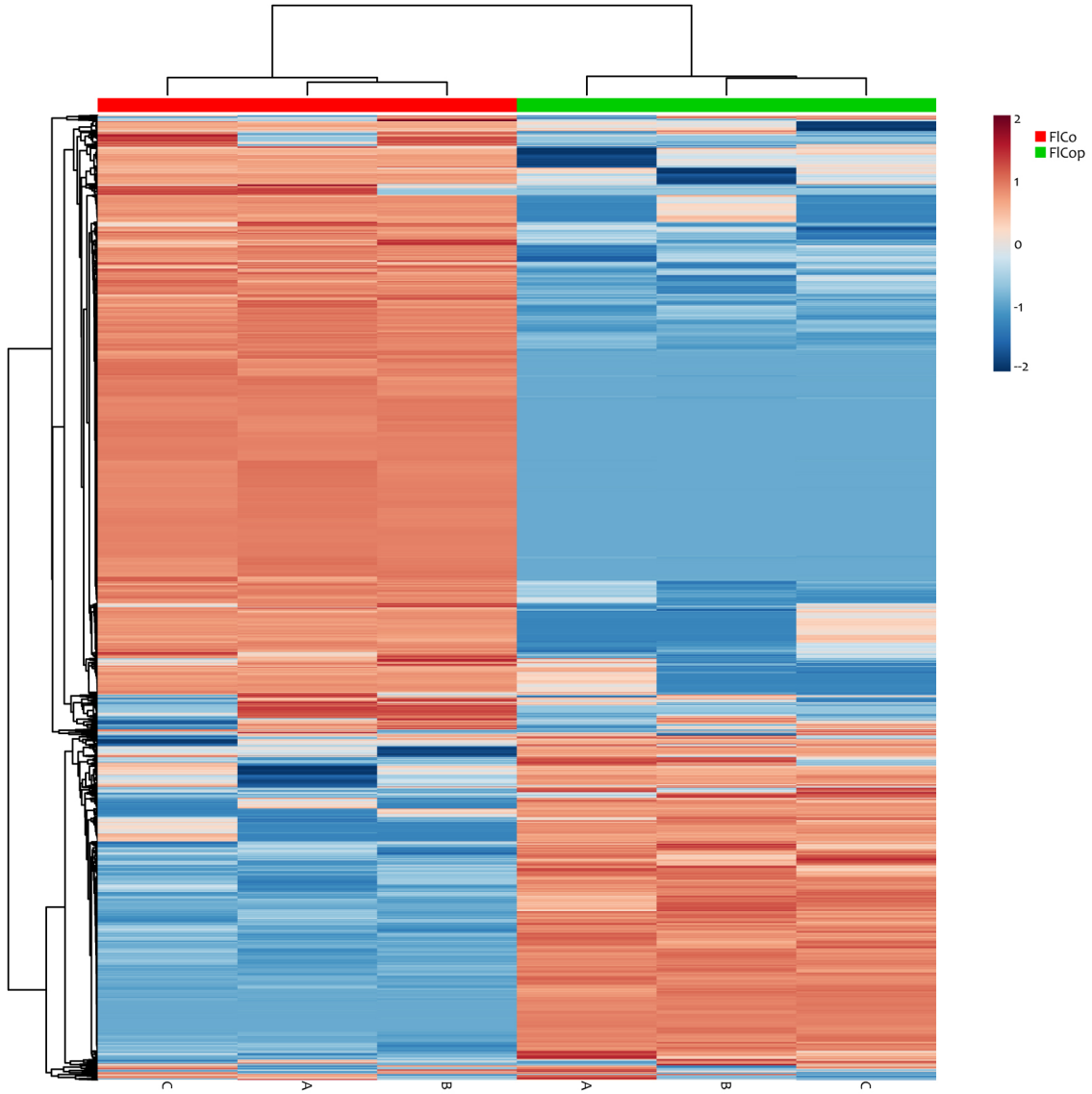


FIG S8.8 | Hierarchical clustering heatmap showing divergent metabolomic profiles after 24h incubation of cheetah faecal inoculum with non-hydrolyzed collagen (FICo) or predigested collagen (FICop). Both conditions were tested in triplicate (A-C).

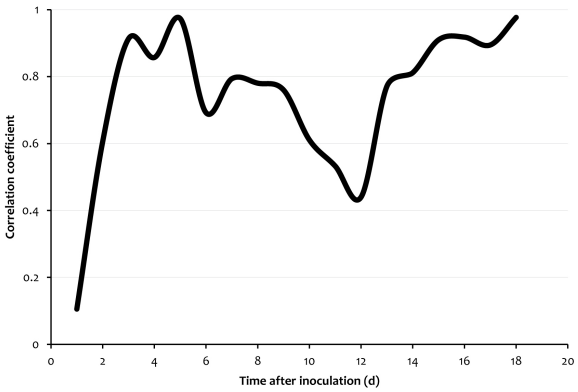


FIG S8.9 | Hierarchical clustering heatmap showing divergent metabolomic profiles after 24h incubation of cheetah faecal inoculum with non-hydrolyzed collagen (FICo) or predigested collagen (FICop). Both conditions were tested in triplicate (A-C)

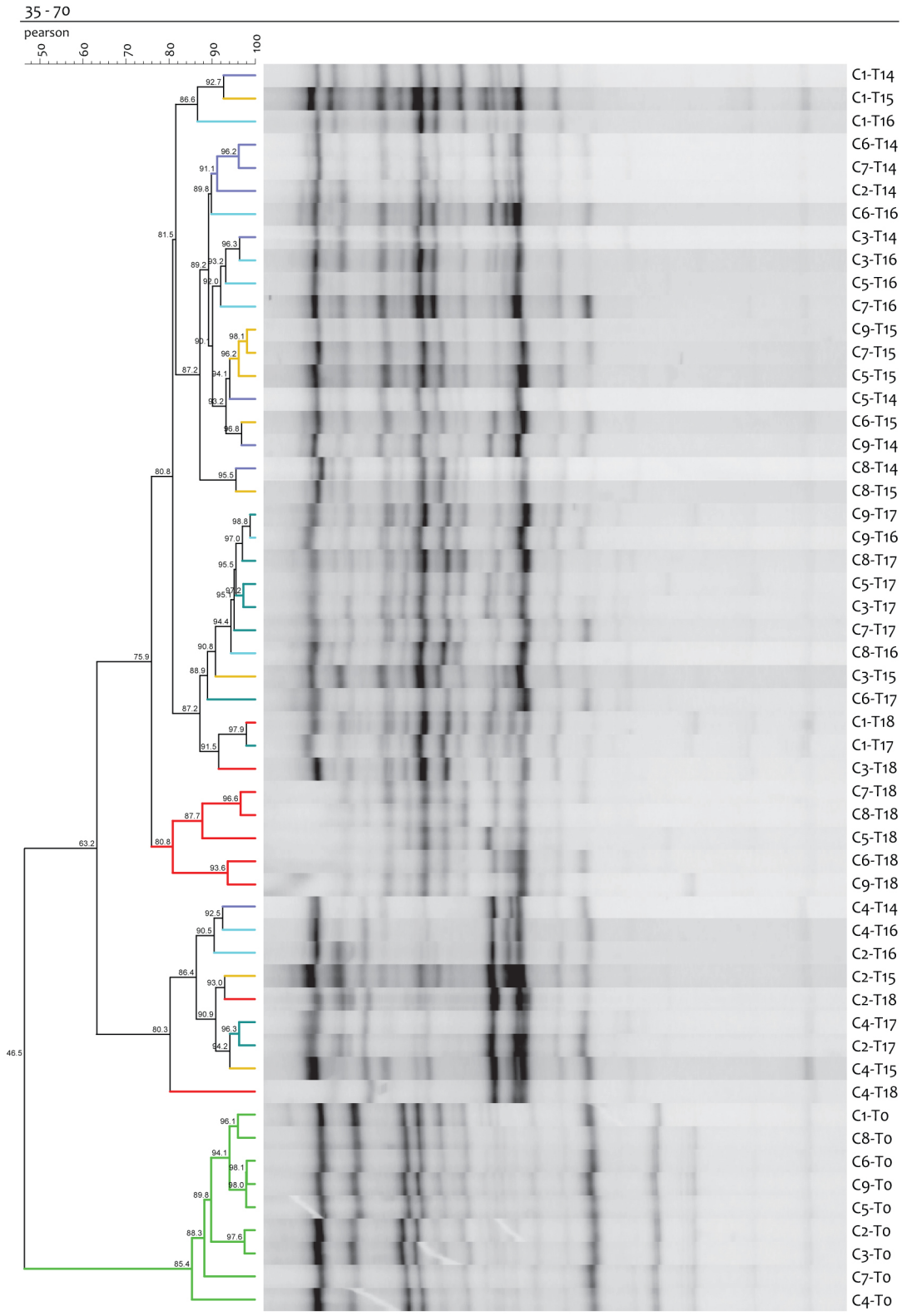


FIG S8.10 | Dendrogram based on Pearson and UPGMA correlations of the DGGE profiles from 9 colon-simulating reactors during stabilization period.

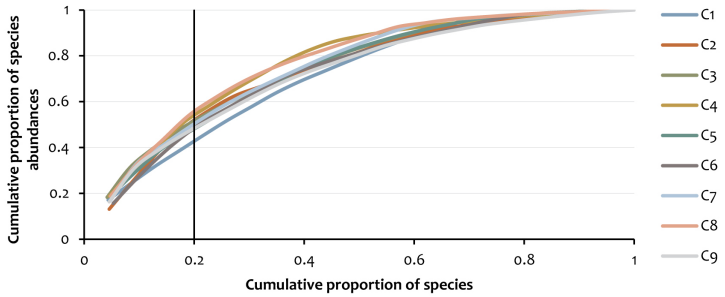


FIG S8.11 | Pareto-Lorenz curves of nine colon-simulating vessels (C1-C9) during the stabilization period (18 days) after inoculation with cheetah faecal inoculum. Functional organization (FO) parameter is evaluated at the 20% level of the x-axis.

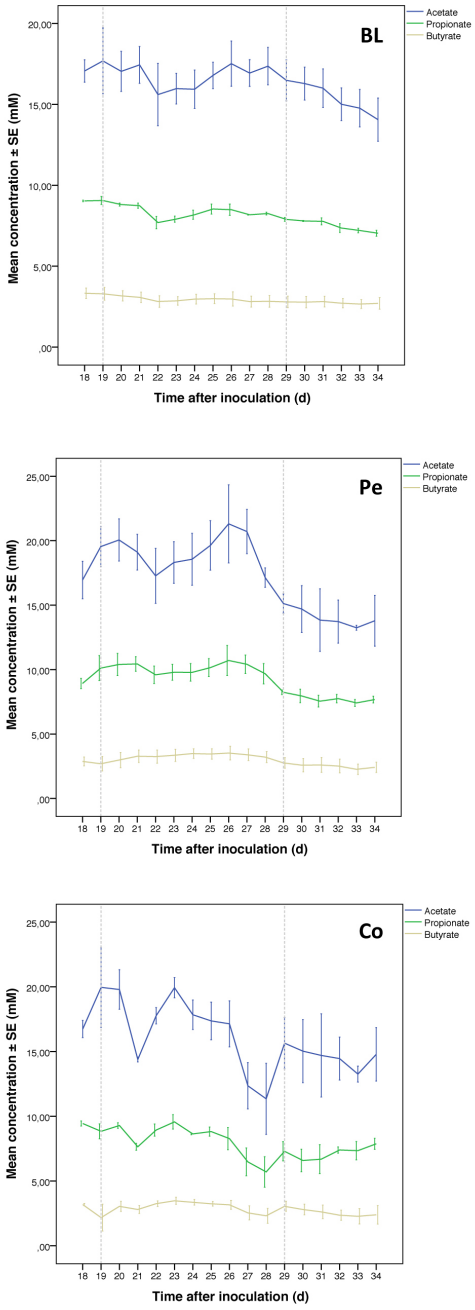


FIG S8.12 | Production of the three main SCFAs in an adapted SHIME model simulating the feline GIT during treatment (T19-T28) and washout period (T29-T34) depicted per treatment group. Each treatment was given to three vessels of the dynamic *in vitro* gut model. BL = control feed, Pe = feed supplemented with Peptan B, Co = feed supplemented with non-hydrolyzed collagen

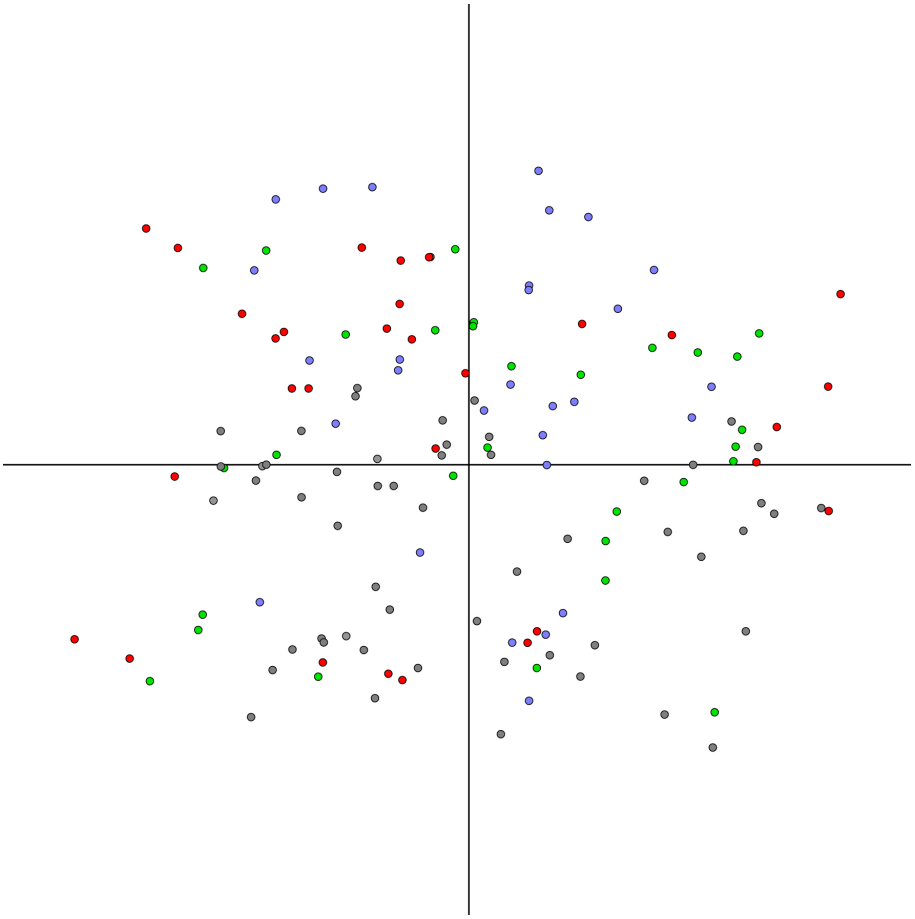


FIG S8.13 | Principal component analysis (PCA) of the V3-16S rRNA DGGE fingerprints of samples obtained from a dynamic *in vitro* gut model simulating the feline GIT. Samples were taken daily during a 10-day treatment period from 9 colon vessels (C1-C3: control, green; C4-C6: Peptan B supplementation, red; C7-C9: non-hydrolyzed collagen supplementation, blue) and during 5 days of washout (C1-C9: control, grey)

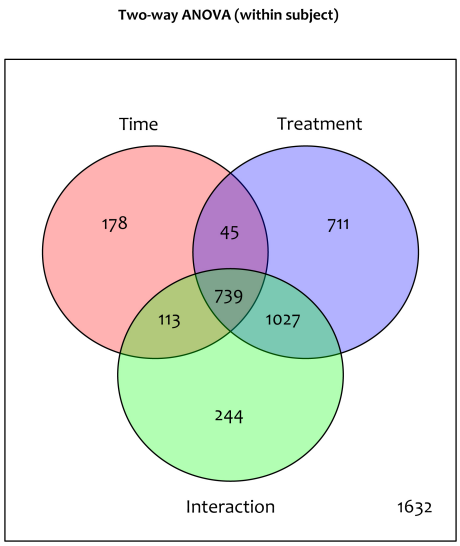


FIG S8.14 | Venn diagram summary of results from two-way ANOVA conducted for each individual metabolite

PART V | REFLECTIONS & PERSPECTIVES

9 REFLECTIONS

9.1 Getting to the core

9.1.1 What's in a name? Defining the nature of a phylogenetic core across individuals

As the field of microbial ecology matures, efforts to define a rigorous framework for understanding gut microbial community structure, function and evolution are hampered by the development of conflicting concepts that apply specifically to host-associated microbial communities. The concept of a phylogenetic core, typically defined as a suite of members shared among microbial consortia from different individuals of the same host species [172], has been challenged by the increasing depth offered by every next-generation sequencing technique. Dissection of gut ecosystems at such high levels of resolution has revealed pronounced intra- and inter-subject variability and made scientists focus on the plasticity and dynamics of the gut microbiota and the underrepresentation or even the lack of core species [89,465]. However, host-microbiota symbiosis relies on the favourable selection of mutualists and their stable maintenance over time in the host to establish a beneficial partnership [466], which makes the non-existence of a core highly unlikely. The ultimate goal here is to consider various aspects to determine a phylogenetic core across individuals. This not only includes community membership (*i.e.* presence/absence), but also relative abundance (*i.e.* dominance, rarity), persistence (*i.e.* shared members over time within and across communities) and phylogeny (shared lineages across communities). During the course of this work, many of these aspects have been taken into account when assessing the gut microbial diversity of captive cheetahs, including the fact that the size of the core microbiota can also be underestimated due to undersampling. The latter is particularly challenging in a research field that concentrates on endangered and/or free-ranging exotic species (**§9.2**). Despite caveats associated with conceptual definitions, uncovering phylogenetic cores still provides an important framework to increase our knowledge on the processes and conditions shaping the gut microbiota (e.g. host-related and environmental cues) and to guide manipulation of these communities to achieve desired outcomes.

9.1.2 Evidence for a mutual and stable phylogenetic core gut microbiota among captive cheetahs

At the onset of this study, in-house laboratory protocols for clone library construction were readily available and provided a first tool for screening the hithertho unknown microbial community diversity in faecal samples. As a result, sequence analysis of 16S rRNA gene clone libraries prepared from samples from two cheetahs provided the first taxonomic picture of the faecal microbial diversity in captive cheetahs (**Chapter 4**). Taxonomic analysis revealed a microbiota clearly dominated by Firmicutes (94.7%), followed by a minority of Actinobacteria (4.3%), Proteobacteria (0.4%) and Fusobacteria (0.6%). Within the Firmicutes, the majority of the phylotypes belonged to *Clostridium* clusters XIVa (43%), XI (38%) and I (13%). Being aware of the fact that these insights encompassed only two cheetahs and that a limited number of clones were processed, these first results were further substantiated (i) by expanding the methodological portfolio with DGGE community profiling, qPCR (**Chapter 7**) and Illumina MiSeq sequencing (**Chapters 6 & 7)**, and (ii) by expanding the sample set with faecal samples from 48 cheetahs housed at 13 different zoos. Taking into account presence of phylotypes across individuals, Illumina MiSeq sequencing revealed a larger share of Actinobacteria (11.5%) and *Lactobacillaceae*. However, when taking into account relative abundances as well, 2 phyla (Firmicutes and Actinobacteria), 3 orders (Coriobacteriales, Lactobacillales and Clostridiales) and 4 families (*Coriobacteriaceae*, *Enterococcaceae*, *Clostridiaceae* and *Lachnospiraceae*) contributed to more than 80% of the reads at the respective taxonomic levels, indicating their potential membership of the core microbiota. The possible contribution of low-abundance taxa to this core is more difficult to assess. Although these taxa are not considered to be core members based on the fact that they were generally detected in a smaller number of samples, they still may represent stable members of the community occasionally falling below the detection threshold due to temporal fluctuations. Consequently, the issue of temporal dynamics must be considered when on the hunt for a core. To this end, 23 days after the start of this PhD work, the first samples of a long-term monitoring study in 5 cheetahs were collected. One might debate the small number of animals included, but the length of this study (3 year, bimonthly follow-up) probably outweighs its limited sample size and has provided first-ever insights in the faecal microbial dynamics of an exotic carnivore (**Chapter 5**). On the other hand, time series samples based on bimonthly snapshots of the community structure are not comparable to continuous monitoring of kinetics, which is not feasible *in vivo*. In addition, the use of different methods, each of which are characterized by specific levels of sensitivity and specificity, may contribute to different conclusions. This can be an important issue regarding the interpretation of our results and comparison with other studies. The core set of key players belonging to *Clostridium* clusters XIVa, XI and I varied over the sampling period with only 0.59, 0.72 and 0.87 log10 CFU/g, respectively. Additionally, 65.34% of shared Bcl in community profiles were retrieved after 1 year and 85% of abundant OTUs was shared between consecutive sampling dates within each animal. The overall community profiles of faecal samples from cheetahs displayed only 23.3-25.6% change, mostly the result of variation in proportional composition at the phylotype level and not so much of differential species richness (**Chapter 5 & 6**). Our data answer to the perception of a temporally stable faecal microbiota, which is largely based on the observation that intra-

individual differences in time are much less pronounced than inter-individual variations [69,87]. Stable configurations of the gut microbiota should in fact be regarded as resilient stable states fluctuating with a certain degree of variation around the equilibrium [88]. Allowing for a degree of variability is the gut microbiota's powerful key for survival and success. Rigid constellations stand no chance to survive, neither in natural nor in social environments.

Defining temporal dynamics inevitably leads to the question what can be considered 'normal temporal variation' and at what phylogenetic level this should be considered? From what point on should fluctuations be considered 'abnormal'? Next, more in-depth questions can be defined. For instance, should temporal variation be considered only at phylogenetic levels, and how do compositional perturbations correlate to functional levels (e.g. gene expression, metabolite profiling)? If different microbial constellations contribute to similar metabolic homeostasis (*i.e.* functional redundancy), should we not better shift our focus towards defining functional cores across hosts (**§9.3**)? Keeping this in mind, our observational diversity studies are restricted to only one side of the core *i.e.* phylogenetic diversity. This is, in part, due to the lack of microbial metabolic frameworks for many mammalian hosts (especially carnivores such as the cheetah) and the primary focus on microbial diversity in long-term studies (mostly in humans), consequently providing the only robust framework for comparison [87,89,467].

9.1.3 Hinting at factors that shape the gut microbiota in captive cheetahs

Cataloguing faecal microbial communities in terms of structural and temporal diversity is only a first step in the exploratory trip to better understand the symbiotic relationship between gut microbes and their hosts. After all, an increased understanding of how much the microbiota varies with external factors will inform strategies for manipulation of the gut microbes. Knowing that they are deeply integrated into almost all aspects of mammalian life (**§2.3**), we're already starting to see glimmers of future treatments. Large-cohort comparative studies in humans are conducted to link environmental and host-related factors to microbial diversity and function [468]. Such large cross-sectional studies that can factor out important variables are almost impossible to conduct with endangered animals (**§9.2**). **FIG 9.1** summarizes factors, deduced from literature and our sampling metadata, which might influence the composition and/or function of the cheetah gut microbiota. Additionally, complex interactions between symbionts and invading pathogens will also contribute to (unpredictable) community shifts, which further complicates linking factors to community variation.

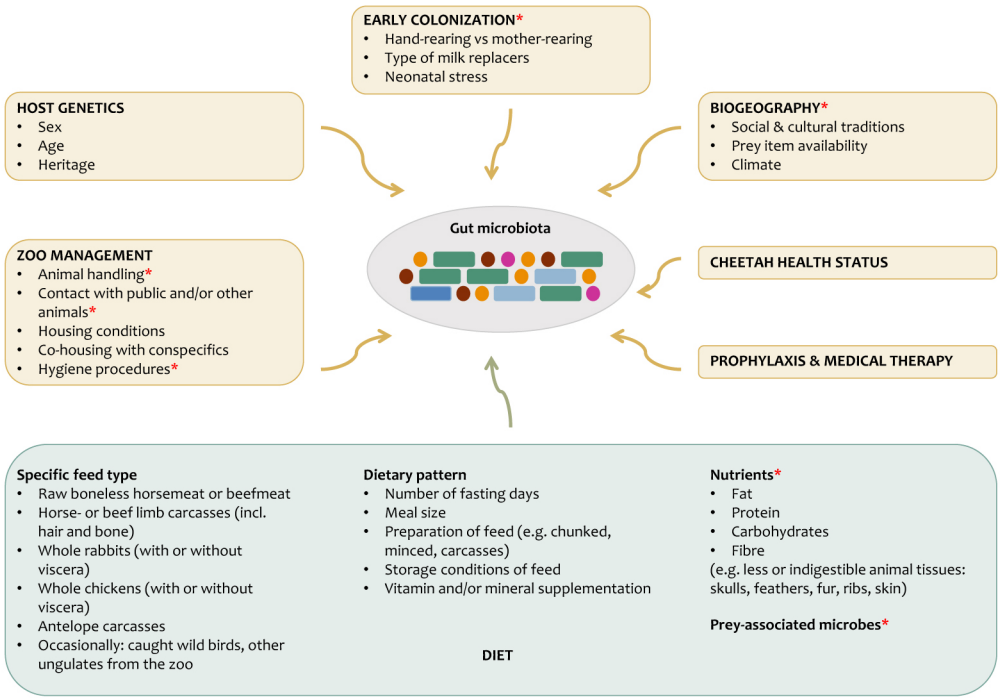


FIG 9.1 | Factors potentially influencing the composition and/or function of the captive cheetah gut microbiota, with special focus on diet *= Metadata for which we had no or limited information

It is important to note that none of the factors were controlled for or changed during sampling. Nor did the design of the cross-sectional or longitudinal studies allow for an appropriate fit of multivariate generalized linear models to associate microbiome data and all metadata (**§9.2**). Investigating the influence of specific factors on gut microbiota composition was beyond the scope of this work, but the continuous monitoring of several metadata (**FIG 9.1**) provided clues to potential important factors. One such factor is **diet type**, although one has to keep in mind that this is likely to be confounded by many other factors (e.g. prey quality, amount and availability of feed, feeding preferences of the animal etc.). Upon clustering of animals fed either raw meat diets (>90% chunks of horse or beef meat without bone), mixed diets (whole rabbits regularly interspersed with chunks of beef meat with or without bone) or whole chicken diets, the cheetahs receiving the latter clustered separately, despite being housed at two different locations (**Chapter 7**). Another important factor is **health and disease status** of the animal. One cheetah suffered from intermediate periods of vomiting and diarrhoea during the monitoring period, which appeared to be associated with changes in two microbial key groups, *i.e.* increase in members of *Clostridium* cluster I and a decrease in *Clostridium* cluster XIVa members (**Chapter 5**). Two other cheetahs suffered from chronic renal failure, a significant cause of mortality in captive cheetah populations [34]. Both had been for more than one year on a special diet of minced meat and the commercial formulations Royal Canin Renal Kibble and Ipakitine which lowers the absorption of phosphates from the intestine. Samples of both cheetahs were significantly depleted in *Clostridium* cluster XIVa (data not shown), which could be potentially due to the drastically changed dietary pattern or the disease or a combination thereof. However, no other parameters were measured at the time to

determine, for example, the severity of disease in these animals. It is likely that disordered health as well as dietary patterns may significantly influence gut microbial composition, as shown in data from other host species [96,265,292,296,469].

Another critical point concerns the approach to assess health status of wild animals such as the cheetah housed in captivity. During veterinary training, one is told to quickly approximate an animal's health status (prior to further examination) by these 6 aspects: inspection, percussion, palpation, auscultation, hydration and temperature registration. Obviously, the hands-off working policy generally promoted in zoos requires alternative measures. Health status of the cheetahs included in this study was assessed by specimen reports which included detailed medical records (e.g. anthelmintic prophylaxis, parasite control of faecal samples, therapeutic treatments, changes in diet and any observations of abnormal behaviour) and prolonged observation during sampling (minimum 3h). This observation included inspection of body condition, physical activity and interaction with conspecifics, defaecation behaviour, feeding behaviour and appetite next to scoring of the faecal samples for presence of parasites, blood, particulate matter, undigested feed components and consistency following a standardized faecal scoring system [39]. Additionally, housing conditions and dietary regimes were registered. When working with zoo animals, one should keep in mind that many of these aspects may be biased due to the lack of standardized measures and thus rely on the observer's criteria. To this end, recently published work [39] highlights the importance of large cross-sectional surveys enclosing many zoological institutions to reveal associations between different factors, redefine factors and establish a more universal method of health status assessment in exotic species housed in zoological settings.

9.1.4 How wild does it get?

When presenting this work at conferences, one of the most frequently asked questions involved the comparison of the gut microbial composition between captive and free-ranging cheetahs. The interested questioner usually completed with the assumption that "there must surely be a big difference, probably pointing towards the negative impact of captivity." This coloured idea portrays the gut microbial constellations in black-and-white, nourishing false dichotomies of the good and the bad, or the better free-ranging and worse captive animal settings. Near the end of this PhD work, we could lay hands on faecal samples collected max. 24h after defaecation from free-ranging Namibian cheetahs. Preliminary screening of microbial communities with DGGE fingerprinting and group-specific qPCR assays revealed on average an equally diverse microbial community compared to captive cheetahs. Although holding the same set of key players, these varied more in relative proportion within the free-ranging cheetahs. In addition, the abundance of members of *Clostridium* cluster XI was significantly lower in free-ranging cheetahs (**Chapter 7**). Potentially this can be attributed to different protein content and quality of their respective diets as *Clostridium* cluster XI has been positively correlated with dietary protein content [265]. Interpretation of these data warrants caution because it is tempting to attribute the observed differences to intrinsic factors of free-ranging and captive populations, and thus state that core gut microbial composition is different. Firstly, unequal sample size between both

populations may have provided a distorted picture of the range in bacterial proportions. Secondly, temporal variation of microbial community structure in free-ranging cheetahs is unknown. It is likely that seasonal variation in prey availability and quality, hunting skills, competition with other predators, etc. may impact gut microbial community structure and dynamics. Many more samples have been collected over a timeframe of 2 years from the free-ranging cheetahs under study. It might be worthwhile to first have a closer look at these and characterize the core community in terms of diversity and stability. However, based on what we currently know, the potential interactions among mammalian host habitat, gut microbiota, and host fitness, are significant and studies of wild animals are a critical next step for examining the co-evolution of host and gut microbe [432].

What constitutes a healthy gut microbiota in either captive or free-ranging cheetahs is both an intriguing and complex question. From an operational point of view, it could be stated that a healthy gut microbiota is the microbiota composition as it can be found in healthy cheetahs. However, substantial inter- and intra-individual variation obscures precise relations between host health and the presence and relative abundance of specific microbial communities. In fact, it is important to maintain a state of homeostasis between host and gut microbiome, and different microbial constellations can succeed in this. Insights into core microbiomes should thus be enriched by –omics approaches, thereby extending the concept of the core beyond taxonomically defined membership to community function and behaviour (**§9.3**).

9.1.5 A core signature for the gut microbiota of carnivores and carnivorans

Based on literature, the carnivore-associated microbiota seems to be characterized by the enrichment of lineages from the phylum Firmicutes, especially *Clostridium*, *Blautia*, *Coprococcus* and *Enterococcus*, and a dearth of Bacteroidetes, *Ruminococcaceae* and *Bifidobacteriaceae* compared to other mammalian species (**Chapter 3**). Our data substantiate these prior observations and especially connect with microbiome data obtained from other strict carnivores within the Carnivora such as polar bears, hyaenas and lions [253,255,332]. This is in contrast to lynxes, dogs and grizzly bears which hold a larger share of Bacteroidetes and Fusobacteria [261,263,332]. One study suggested the intriguing possibility that the lynx microbiome evolved to digest not only the meat of its prey, but also the plants constitutive of its prey's diet [263]. Potentially, the inclusion of less-digestible plant-based feed components differentiates more omnivorous from strict carnivorous members of the Carnivora. Likewise, indigestible animal tissues in the diet may act as animal fibre in strict carnivores feeding on whole prey [49,52]. Differences in dietary items have the potential to change the gut microbiota through the introduction of novel phylotypes or alterations in available nutrients, and therefore substrates available to bacterial members. In theory, differences could exist between different strict carnivores in their adaption to such difficult-to-digest components. Small carnivore species mostly consume whole prey, including fur, feather, skin, (rib)bones and connective tissues as well as muscles and viscera. In contrast, larger carnivores feed on prey within the range of their body mass and consequently can afford to

selectively feed on the more digestible parts [470]. One could hypothesize that smaller-sized carnivores might be better adapted to consumption and host microbiota fermentation of less digestible diet components. This suggests that broad sampling across a wide range of carnivore species might be necessary to gain a full understanding of the core microbial diversity in a strict carnivore.

However, the intertwining of diet and host phylogeny has been notably illustrated in the order Carnivora, and at shallower taxonomic levels microbial communities of conspecific hosts tend to be more similar to each other than to those of different host species sharing the same diet type (**Chapter 3**). To verify this at a deeper level, we used Illumina MiSeq as a next-generation sequencing approach to compare the cheetah's gut microbial diversity with data obtained for a limited number of domestic cats, which are also members of the *Felidae* and frequently used as models for strict carnivorism (**Chapter 7**). The assumption that a domesticated felid, usually feeding on commercial petfoods, still represents all facets of carnivorism warrants caution. Commercial petfoods are highly variable with regard to their format, macronutrient composition, fibre content and inclusion of functional ingredients (e.g. prebiotics and probiotics), which may impact feline gut microbial composition (reviewed in [261]). For example, increased abundance of *Megasphaera* has been observed in cats fed dry diets (33% protein, 11% fat and 46% carbohydrates on dry matter basis) compared to wet diets (42% protein, 42% fat and 5% carbohydrates), and high-carbohydrate diets favoured the growth of representatives of Erysipelotrichales [298,471]. Our findings included a significantly higher proportion of *Veillonellaceae* (*Megasphaera* spp.) and *Erysipelotrichacae* (*Catenibacterium* spp.) in faeces of domestic cats feeding on commercial kibble diets compared to captive cheetahs. The divergent microbial profile of the single cat in our study that was feeding only on raw meat further nourishes the expression "you are what you eat". Despite their broadly similar carnivorous nature, different dietary habits of different *Felidae* may set their microbiomes further apart than initially thought. Consequently, diet-related microbiome studies should not only include different commercial diets but also raw meat diets, next to cross-sectional observation studies with broad sampling of different feline hosts (including feral cats as well). The combined data will further provide insight into the potential core members of the *Felidae* and whether or not the domestic cat is the misfit.

9.2 Challenging sampling

Studies in vulnerable and (critically) endangered animal populations are associated with a variety of sampling challenges including access to animals (e.g. free-ranging cheetahs) and the collection and preservation of samples. Many of these challenges can be overcome through collaboration between ecologists with established field projects, researchers with established analysis protocols and international captive cheetah population managers. For microbial analyses, faecal samples are generally the easiest type of sample to obtain from free-ranging and captive cheetahs. Because faecal samples will normally come into contact with the environment, sample contamination is likely to occur. Care should be taken to collect samples with sterile equipment and avoid obvious contamination by only

taking parts of the sample that have not come into direct contact with the environment. Especially when cheetahs are mainly fed whole prey, faecal material is very heterogeneous and includes undigested dietary particles and dry scat consisting only of hair. Influence of sampling location within a faecal sample on genomic DNA quality has been described [472]. Consequently, we carefully considered at the onset of this work sampling location within faeces and compared DGGE fingerprints from different locations within a sample (**FIG 9.2**). In general, richness (estimated by number of bands) decreased when scratching 1 g from the outer side of the faecal sample or the dry hairy scat. Therefore, we decided to change in-house laboratory homogenisation protocols and included 25 g as starting material, which was obtained from all parts of the faecal sample.

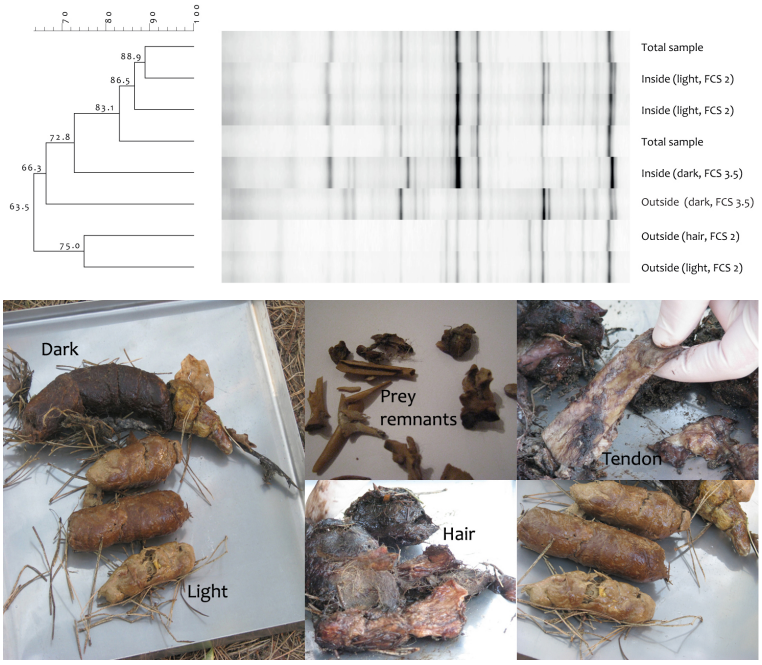


FIG 9.2 | Cluster analysis of DGGE fingerprints based on the Dice coefficient of the total bacterial community in different locations of the same faecal sample from an adult, apparently healthy, male captive cheetah

Immediate freezing at -20°C and long-term storage at -80°C is the preservation method of choice for microbiome studies [473]. If collection is aimed at culturing, anaerobic conditions need to be immediately assured at 4°C [173,175] (**§2.4.2**). However, ice packs, liquid nitrogen and/or freezers are not always available at remote field sites. Additionally, even if freezers are available, not all commercial shippers allow the use of dry ice so the risk of samples thawing or going through freeze-thaw cycles during transport must be considered, especially when collecting samples in the field or at zoological institutions located at a certain distance from the laboratory. Alternatively, the use of liquid-based preservatives (e.g. RNAlater®) has been evaluated and can be of use depending on the purpose of analyses, but it still remains ideal for faecal samples to be frozen and for nucleic acids to be extracted as soon as possible [474,475]. However, batch processing of faecal samples is not always feasible, since sample collection and monitoring defaecation in low-numbered captive or free-ranging populations is time-consuming. In addition, if we were to compare different *Felidae* populations (e.g. lion, lynx etc.), we must take into account that defaecation in some carnivorous species can take up to several days.

As mentioned previously, large-cohort studies are needed to sample a variety of populations and obtain sufficient data to separate and identify potential host-related and/or environmental factor effects on the gut microbiota. The design of our cross-sectional or longitudinal studies did not allow for an appropriate fit of multivariate generalized linear models. Apart from structural zeros (prediction that does not exist e.g. free-ranging cheetah receiving commercial raw meat diet), too many predictions (resulting from possible combinations between factors) were not fulfilled. Alternatively, one can consider grouping different factors together, though this may again obscure individual factor effects. It is hard to piece out correlation and causation, and to this end, parallel research in controlled laboratory settings (e.g. *in vitro* [dynamic] gut models) may contribute significantly to microbiome research with exotic animals such as the cheetah and overcome some of the limitations inherent to *in vivo* studies.

9.3 Metabolites: footprints of host-microbial co-metabolism

9.3.1 The critical omics puzzle piece

The metabolic crosstalk between gut microbiota and mammalian host is accompanied by many channels of chemical information exchange, mainly in the form of low molecular weight metabolites, peptides and proteins. Some of the main chemical classes involved include SCFAs, bile acids, choline metabolites, indolic derivatives, phenolic, benzoic, and phenyl derivatives [100], but in all probability there are many more still to be discovered. Whereas metagenomics, metatranscriptomics and metaproteomics present the potential for a biological outcome, metabolomics provides data on the true endpoints of the phenotypic response. Consequently, it eliminates the need for any assumptions to be made about the origin of a phenotype and provides insight into functional metagenomic activity. However, the wide array of metabolites is the result of a dynamic chemical dialogue between host and gut microbes, but also among gut microbes through quorum sensing and cross-feeding [137,476]. Many investigations of gut microbial populations and metabolism aim at monitoring specific end-products of bacterial metabolism (e.g. SCFAs), generally confined to faecal samples or bacterial cultures and isolates. Such measurable outcomes provide some essential -yet limited- insights into a restrained range of microbial activity, but it takes more to decipher the depth of host-microbe symbiotic partnership. Like it was once necessary to move from traditional culturing to culture-independent and next-generation sequencing techniques to acknowledge the depth of microbial diversity, high-throughput metabolomics will be the essential next step to generate multivariate information on a wide range of metabolites in various biological matrices and acknowledge the depth of host-microbiota signalling.

However, the emerging field of metabolomics faces multiple challenges. The simplicity in sample preparation and metabolite extraction is countered by the huge data flood which renders the simple "stare and compare" statistical approaches inadequate. The goal of metabolic fingerprinting (*i.e.* untargetted metabolomics) of samples from our batch- and SHIME-experiments (**Chapter 8**) was to determine relative differences between the metabolomes of two or more conditions to infer a cause-effect relationship. A hallmark of metabolic fingerprinting is the use of **multivariate analysis methods** [477], which we successfully applied to deduce metabolites contributing to class differences. Although we only used the linear projection-based methods of PCA and OPLS-DA, hierarchical clustering analysis and support-vector machine methods can be applied as well [477,478]. Bearing in mind that OPLS have an innate tendency to over-fit models to data and identify class separation in completely random variables [477], model reliability was ensured by internal cross-validation and CV-ANOVA. This revealed different patterns or 'fingerprints' of metabolites in response to the different conditions (**Chapter 8**). However, after downscaling of data to a subset of significantly discriminating metabolites, we encountered another major hurdle *i.e.* **metabolite identification**. In order to find tentative identifications and to obtain functional information, different small-molecule and pathway databases were queried, e.g. comprehensive metabolomic databases [HMDB], metabolic pathway databases [KEGG, MetaCyc], compound databases [KEGG Compound, ChEBI, PubChem] and spectral databases [Metlin, HMDB]. However, the fact that only 8% could be unambiguously identified hampers further biological interpretation of metabolic fingerprints. At present, each one of these databases covers only a fraction of the human metabolome and even less from other organisms. Although quantitative metabolite data from different model organisms are abundant in literature, their integration in global databases has yet to be accomplished [479]. Additionally, microbial metabolites are rather poorly covered by these databases since metabolomics is a relatively new discipline in the field of microbial ecology. Similarly, the lack of reference NMR or MS spectra for all known metabolites makes proper identification of metabolites challenging [477]. However, using metabolomics exclusively for fingerprinting without identifying metabolites that cause clustering of experimental groups will only deliver a classification tool and hypothesis-generation approach, but not directly contribute to biochemical knowledge and understanding of underlying mechanisms of interaction. The real power of metabolomics is realized when both qualitative and quantitative analyses are performed. To this end, data of metabolic profiling (*i.e.* targeted metabolomics) should be used to build databases. Metabolic profiling is a hypothesis-driven approach in which metabolites are selected for specific analysis. Instead of target analysis of SCFA and NH_4, an alternative approach in our studies could have been high-throughput metabolic profiling in batch-experiments targeting a larger number of microbial metabolites with potential biological functions in carnivores (e.g. SCFA, BCFA, NH_4, biogenic amines, indole derivatives etc.) prior to the experiment with the dynamic *in vitro* gut model. Standards of desirable compounds should then be included for metabolic fingerprinting of samples from the dynamic gut model (e.g. this has been done for hydroxyproline). Alternatively, database search results from metabolic fingerprinting (e.g. metabolites from metabolic networks) can be confirmed by comparison

with a reference spectrum and chromatographic retention of the standard. This implies a second round of sample investigations with the UHPLC-Orbitrap HRMS now including a series of standards of the specific compounds. **FIG 9.3** summarizes a number of complementary approaches to broaden the scope and readouts of metabolic investigations.

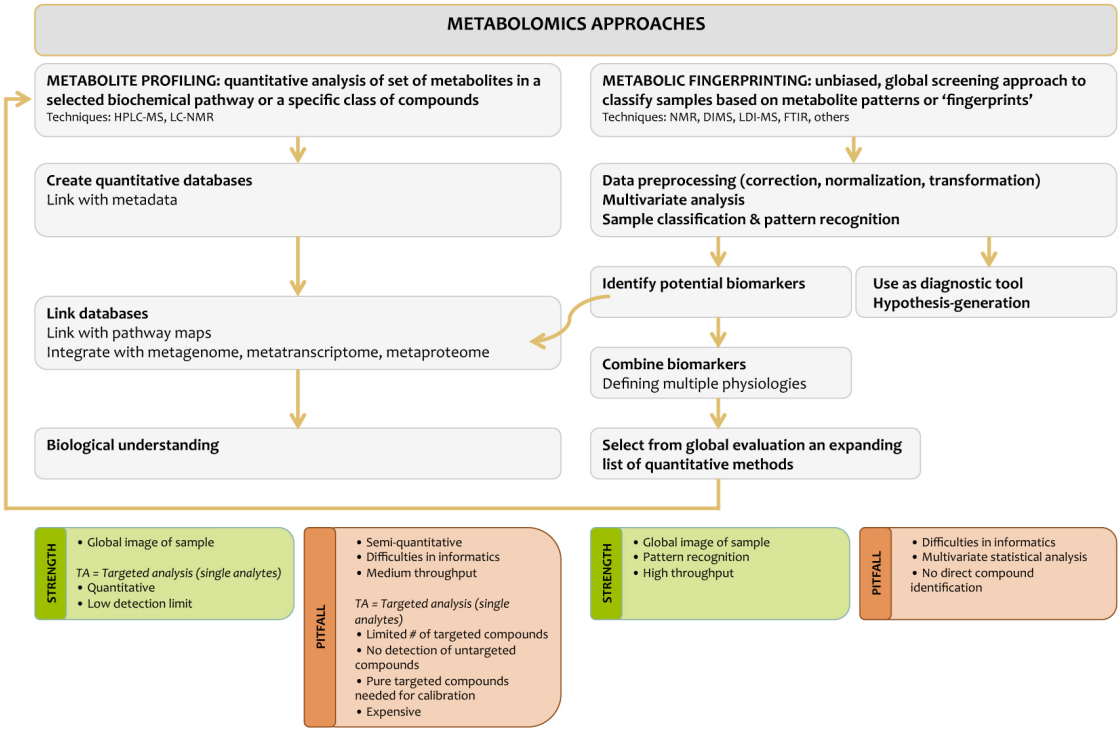


FIG 9.3 | Strategies for complementary metabolomics approaches for intestinal ecosystems

9.3.2 Integrating metabolomics and *in vitro* dynamic gut modelling

Considering that many metabolites present in faeces derive from non-digestible food ingredients, faecal metabolite profiling may be useful to explore the fate of diverse dietary components or diet compositions. However, *in vivo* studies are easily perturbed by any number of host-related or environmental factors. Also the chemical complexity and heterogeneity of metabolites, and the wide dynamic range and short half-lives make it difficult to identify the few chemical features against a large and complex background of metabolites that uniquely relate to dietary components [128]. Additionally, for animal welfare reasons, animal experiments have to be refined and reduced to a scientifically sound essential minimum. To this end, dynamic *in vitro* gut modelling presents an opportunity to gain mechanistic insights in dietary modulation of the gut microbial community prior to dietary intervention studies *in vivo* by controlling as many factors as possible to clarify cause-effect relationships.

Prior to substrate testing, the adapted dynamic *in vitro* gut model requires microbial and metabolic steady-state. The latter has only been assessed by targeted analysis of the main expected fermentative outcome for which some reference values were available (SCFAs and NH_4). Additional metabolic fingerprinting of samples during the stabilization period could have revealed the evolution of the

metabolome over time, potentially gaining more insight in nutrient utilization and cross-feeding processes in communities adapting to the imposed *in vitro* setting and generating a more refined definition of whether or not metabolic stasis was achieved. Additionally, our study lacked metabolite fingerprints of the unfermented medium to confirm differentiation between different substrate conditions was not due to unmodified compounds present in the medium. Prior to substrate testing, one should hypothesize the potential metabolic outcome of substrate fermentation and focus on metabolic profiling of compound groups in short-term static batch-experiments. For example, fermentation of non-hydrolyzed collagen can give rise to collagen-specific building blocks (*i.e.* proline, glycine, hydroxyproline), but, -depending on fermentation rate,- also to SCFAs, NH_4 and a number of biogenic amines. Likewise, targeting the amino acids that make up the composition of Peptan B could confirm whether or not the observed increase in NH_4 and SCFAs was due to microbial metabolism of these specific amino acids (and eventually which ones were preferred). If *in vitro* changes in metabolite profiles correlate with microbial profiles, one could try to identify target species.

However, whereas these *in vitro* models provide mechanistic insights in potential nutritional modulation, this does not necessarily mirror diet-induced changes in microbial composition *in vivo*. Moreover, during *in vivo* dietary intervention studies, host-absorption of metabolites and metabolite biotransformation will likely result in differential faecal metabolite profiles. In this context, combined analysis of the metabolome in different body fluids (e.g. urine, saliva, blood) might be a feasible strategy to establish coherent links between the bioconversion of non-digestible diet ingredients, their bioavailability and their downstream effects and relative importance on host and microbial metabolism.

One important aspect that may contribute to the success of dietary intervention studies *in vivo* is the systematic characterization of the faecal metabolome in different species [480]. Although we have characterized core gut microbial communities in captive cheetahs in which consistent differences with free-ranging conspecifics and domestic cats were observed, we don't know whether these feline species (and to a larger extent carnivores) share a functional core. Characterization of the faecal metabolome may reveal species-specific differences [480]. Additionally, variation in the biochemical composition of faeces caused by age, time or interindividual differences is small compared to variation induced by diseases or nutritional interventions [292,481]. Consequently, metabolic fingerprinting of faecal extracts can provide a baseline for future investigations applying faecal metabolite profiling to monitor the effect of dietary interventions. Moreover, indication of the more stable metabolic components should facilitate the identification of more variable components or potential biomarkers for disease or diet-induced changes.

As such, what should be considered a 'healthy microbiome' should not only be defined by which microbiomes are resident in the gut but by what microbiomes produce and thus how host-microbial metabolic homeostasis is achieved. For wildlife species housed in captivity such as the cheetah, identification and quantification of metabolites with potential beneficial or detrimental outcomes might provide estimates of (sub)optimal (GI) health.

9.4 Where to go from here?

"We now have the technology to begin to understand what van Leeuwenhoek saw under his microscope. The good news is that advances in molecular technology have been the key to opening the door to the detailed analysis of symbiotic partnerships. The bad news is that these advances have also revealed the scale of the task ahead." (M. McFall-Ngai)

The conversation between partners in a beneficial relationship is complex and multi-layered. A prime example thereof is the metabolic crosstalk between mammalian hosts and their gut microbiome. At this point, we are still in the exploratory phase for most mammals and ask questions such as: what microbial constellations reside in the gut of different animals; what are the patterns of the associations of microorganisms with animals; how profoundly have these alliances affected the partners' evolution; and how do symbioses affect other aspects of the partners' biology and vice versa?
This work has focused on the underexplored partnership between a strict carnivore, the cheetah, and the microbial critters in its simple gut. Recent studies in different host species (including domestic cats) have provided compelling evidence that the microbial metabolites (or host-microbial co-metabolites) directly or indirectly exert beneficial effects on host health and that host-microbiome metabolic interactions are profoundly influenced by a broad range of intrinsic (host-related) and extrinsic (environment-related) factors [261,298]. Consequently, much research effort focuses on the modulation of the gut microbiota and its metabolism with the aim of improving (or maintaining) host health. As such, dietary interventions represent one of the promising approaches within reach and one of the most practicable in management of wildlife species housed under human care. In order to leverage information about the cheetah gut microbiota effectively to dietary intervention and assessment of health status, several knowledge gaps remain to be addressed.

Our monitoring study revealed long-term temporal stability in adult captive cheetahs. However, it would also be interesting to conduct microbiome studies across specific age categories. Cheetahs in captivity may live up to 17 years, with an average lifespan of 8-12 years, while in the wild they mostly reach not more than 8 years. Geriatric cheetahs in captivity are more prone to gastrointestinal and metabolic disorders and chronic kidney failure [34]. In this context, age-related changes in functionality and diversity of their gut microbiomes may provide valuable information that adds to a deeper understanding of their health status. In this context, metabolite fingerprinting may reveal potential biomarkers of disease or suboptimal health. Likewise, early colonization patterns are believed to have a profound impact on the development and maturation of the immune response. Given the fact that breeding of cheetahs is challenging and cub mortality is still high (26% of the cubs that are born in captivity die before reaching 6 months of age) [19], monitoring bacterial succession in cubs and evaluating the effect of diet (e.g. milk-replacers, introduction of meat) and different rearing strategies (e.g. hand-rearing vs mother-rearing) may improve cub health and neonatal survival.

To assess the impact of different diet types and their respective dietary components on the cheetah's gut microbiota, global sampling across divergent dietary strategies may provide new insights into the extent or limits of variation of the cheetah's gut microbial composition. Our study was limited to European zoological facilities, which mainly feed a wide range of different diets (raw meat to whole prey and mixtures thereof) and for which more thorough evaluations of nutrient digestibility are needed. On the other hand, North American facilities mainly feed commercially prepared raw beef and horse meat for which nutrient digestibility has been described in more detail [306,353]. However, microbiome data for cheetahs on these specific commercial diets remain as good as non-existent, but likely provide an interesting case for comparison to our datasets.

The use of *in vitro* dynamic gut models provides undoubtedly an opportunity to assess specific dietary substrate-microbial metabolite associations. However, our model warrants further validation through the incorporation of different inocula from different hosts and fine-tuning of parameters. Additionally, it seems interesting to also evaluate the sugar-depleted protein-rich feed with inocula derived from domestic cats.

To circumscribe the gut microbial diversity and functional configurations inherent to *Felidae*, deeper sampling and analysis will be required including different feline hosts. Also other sample types e.g. mucosal biopsies and digestive fluids may be collected during surgery or routine health check-ups of large captive felids under anesthesia. However, we cannot just choose an animal and describe everything in its gut without considering the internal and external forces shaping its microbial community. To this end, concerted efforts are required and the building of large databases including different types of microbiome data and a wide array of metadata. Recently (May 2015), a team of researchers joined forces to look at the microbiome of all feline species, from big to small, house cats to wild cats, spanning a wide range of healh conditions and habitats. Analogous to the Human Microbiome Project, the Kittybiome project (www.kittybiome.com) aims to map the feline microbiome in all its facets. This may be just the kick-start needed for an exciting trip to the innermost secrets of the *Felidae*.

PART VI | IN A NUTSHELL

TAKE HOME MESSAGE

The consortium of symbiotic gut microbes in a mammalian host can be viewed as a metabolically adaptable, rapidly renewable, compositionally flexible ecosystem varying with multiple host-related and environmental factors. As much as a third of an animal's metabolome has a microbial origin and the circulatory system extends the chemical impact of the gut microbiota throughout the body. The emerging facts about this tiny microbial world within serve as a rebuke to our egos. At our very core, we are less than human and the world belongs to the very small. Charting the gut microbiomes across different mammalian hosts and habitats teaches us to view animals as host-microbe ecosystems. Incorporating an appreciation of the impact of the alliances between animals and microorganisms into our thinking will redefine how to assess animal health and care and may be important to provide insights into certain environmental problems and even conservation issues. Imagine, when an animal becomes extinct, it is likely that some subset of the microbial world – the co-evolved partners of the multicellular organism – will also become extinct. The ability to maintain and successfully breed animals that are threatened with extinction in zoos or protected nature reserves might require more intimate knowledge of their gut microbial ecology and the interrelationships between their native microbiota, diet and nutrient harvest. To achieve the full potential of the gut microbial world in different mammals, a vast array of technical and cultural hurdles must be overcome. Luckily, such challenges in the science of microbial ecology are what render research an exciting arena.

SUMMARY

Quoting one of the mission statements of the World Wildlife Fund, '*protecting wildlife species contributes to a thriving healthy planet*'. From a microbiologist's point of view, however, scientific approaches towards safeguarding the life and well-being of animal hosts facing extinction is inseparable from a better understanding of the microbiota evolutionarily associated with these hosts. It is precisely in this area that the goal of this doctoral work is situated, namely to uncover the largely unknown composition and functioning of the intestinal microbial communities residing in the European captive cheetah population. Bridging this knowledge gap is considered essential to the survival and conservation of this vulnerable to critically endangered species whose successful existence in captivity is impaired by the high incidence of GI and metabolic diseases and low breeding rates (**Chapter 1**). As a global working hypothesis, characterization of their intestinal microbial ecosystem is expected to provide valuable insights into nutritional management and the interrelationship between their native microbiota, diet and nutrient harvest.

They're invisible. They're everywhere. And they rule. The immensely diverse and dynamic microbial consortia residing in mammalian gastrointestinal (GI) tracts represent one of the most intricate forms of a durable, multi-layered symbiotic partnership. Far from being inert passengers, gut-associated microorganisms play essential roles in the most fundamental processes of mammalian life (**Chapter 2, §2.2**). The advent of (high-throughput) DNA sequencing technologies coupled with advances in computational analyses have expanded our ability to perceive the true diversity, ubiquity and functional capacity of these communities, previously gone unnoticed by culturing (**Chapter 2, §2.3**). Evidence for the pivotal role of the GI microbiota in mammalian host homeostasis and susceptibility to disease is mainly derived from data in humans and domestic livestock species. Compared to omnivores and herbivores, very little data exist on the microbiome of strict carnivores such as the *Felidae*. Initial sequencing projects in domestic cats already provided a glimpse of the complex microbiome residing in the simple gut of carnivorous mammals and the profound impact compromised health and different dietary strategies may have on the feline's GI microbial diversity and functionality (**Chapter 3**).

In terms of metabolic substrate supply and availability, the intestinal microbiota is highly dependent upon the fraction of undigested and indigestible food components of the strict carnivore's diet. Nutrition-wise, domestic cats mostly fed commercial petfoods including vegetable ingredients and large exotic felids consuming whole prey including different types of non-digestible animal tissues (e.g. fur, cartilage, skin, bone) are markedly different hosts. Optimal nutritional strategies are a cornerstone of a holistic *ex situ* conservation strategy which assures the well-being of exotic felids housed under human care. An important aspect largely ignored when assessing feeding strategies in exotic felids is the impact of the different diet components on GI homeostasis through altering the intestinal microbiota and its associated fermentation processes. However, the intestinal microbial ecosystem is virtually unknown for most large exotic felids.

This work provides the first taxonomic benchmark on the diversity of the gut microbial ecosystem of captive cheetahs through a multifaceted culture-independent approach using 16S rRNA gene clone libraries, DGGE fingerprinting, quantitative PCR and Illumina MiSeq sequencing (**Chapters 4, 6 & 7**). Phylogenetic profiling data were obtained from faecal samples across the largest European population of captive cheetahs so far investigated for host microbiota studies, including 50 adult animals housed at 13 different zoos from 8 European countries. This body of work revealed that Firmicutes clearly dominated the faecal microbiota of captive cheetahs, followed by a minority of Actinobacteria, Proteobacteria and Fusobacteria. Within the Firmicutes, key players belonged to *Clostridium* clusters XIVa, XI and I. These data were mirrored to data obtained from a smaller set of faecal samples from free-ranging Namibian conspecifics. Despite the obvious difference in environmental factors to which captive and free-ranging cheetahs are exposed, community profiling revealed similar microbial species richness and faecal core members. However, differential clustering among cheetahs fed different diets suggest that diet composition and regimen may trigger proportional differences in specific bacterial groups directly or indirectly affected by changes in supply and availability of preferred substrates. In addition, comparison with data obtained from domestic cats revealed a significantly different microbiota composition between both feline host species, which supports the hypothesis that different dietary habits within members of the *Felidae* may differentiate feline microbiomes to a larger extent than previously thought.

This work also presents the results of the first-ever long-term monitoring study of the faecal microbiota in a feline strict carnivore (**Chapter 5**). Bimonthly follow-up of 5 cheetahs housed at two European zoos over a 3-year period disclosed a remarkable compositional stability of the cheetahs' faecal microbial communities for which compromised host health may be reflected in perturbations of phylogenetic core groups.

Finally, this work also describes the adaptation of the *in vitro* Simulator of the Human Intestinal Microbial Ecosystem (SHIME) model into a dynamic system simulating the cheetah carnivorous gastrointestinal tract under carbohydrate-depleted protein-rich conditions (**Chapter 8**). Under nutritional and physiological conditions that approached those of the cheetah gut, microbial communities reached compositional and metabolic homeostasis after two weeks, and this steady-state was maintained for one month in the adapted *in vitro* model. Upon stabilization, the model was evaluated for its application to study nutritional modulation of the cheetah's intestinal microbiota and its associated fermentation processes by using collagen as a test substrate. The metabolomic fingerprinting workflow for *in vitro* fermentation samples was further evaluated and offers a window to study true endpoints of bacterial metabolism and a tool to assess metabolic crosstalk between mammalian hosts and their gut microbiome.

The author's ultimate prophesy would be that the hitherto uncharted inner microbial world of captive cheetahs mapped in this doctoral work lays the foundations to address a next set of research questions on the fascinating and complex interplay between host, microbiota and diet in these 'greyhounds of the desert' and other wildlife species on the verge of extinction.

SAMENVATTING

We zouden het niet beter kunnen verwoorden dan met de missie van WWF, '*protecting wildlife species contributes to a thriving healthy planet*', wat zoveel wil zeggen als: het redden en beschermen van de biodiversiteit is essentieel voor het behoud van onze planeet. Door het wetenschappelijk oog van de microbioloog is behoud en welzijn van bedreigde diersoorten onlosmakelijk verbonden aan het beter doorgronden van de onafscheidelijke gezellen die zoogdieren met zich meedragen als resultaat van miljoenen jaren van co-evolutie nl. hun microbiële gemeenschappen of microbiota. In dat onderzoeksveld situeert zich dit proefschrift dat tot doelstelling heeft de grotendeels onbekende samenstelling en functie van de darmmicrobiota van de Europese populatie cheeta's in kaart te brengen. Deze kwetsbare exotische katachtigen planten zich niet gemakkelijk voort in gevangenschap en zijn gevoelig voor talloze darmaandoeningen en metabole ziektes (**Chapter 1**). Kennis vergaren over hun gastro-intestinaal (GI) microbieel ecosysteem kan dan ook een sleutelelement vormen in een verbeterd behoud van deze bedreigde diersoort. Mede door het ontrafelen van de relatie tussen de darmmicrobiota en het dieet van de dieren kan het nutritioneel management van deze strikte carnivoren verder gestuurd worden.

Ze zijn onzichtbaar. Ze zitten overal. En ze zijn de baas. De ontzettend diverse en dynamische microbiële gemeenschap die zich verschuilt in de darmen van zoogdieren is het mooiste voorbeeld van een doorgedreven, complexe vorm van samenleven tussen verschillende organismen. Deze bacteriën zijn dan ook allesbehalve toevallige passanten, maar liggen aan de basis van levensbelangrijke processen in hun gastheer (**Chapter 2, §2.2**). Met de opkomst van DNA sequencing en de vooruitgang in de bio-informatica is de microbiologische omvang, complexiteit en functionaliteit pas echt duidelijk geworden (**Chapter 2, §2.3**). Wat we voorheen nog niet konden (be)vatten met de kweek van bacteriën, slaat ons nu met verstomming: de cruciale rol van de darmmicrobiota in het behoud van de gezondheid van hun gastheer en diens gevoeligheid voor ziektes. Merendeel van de kennis hierover komt voort uit studies met een focus op mensen of nutsdieren. In vergelijking met omnivoren en herbivoren bestaan er maar weinig data over de darmmicrobiota in strikte carnivoren zoals katachtigen (*Felidae*). Een aantal sequentiestudies lichtten al een tipje van de sluier op en toonden aan dat ook de huiskatten een complex microbieel ecosysteem herbergen dat sterk beïnvloed wordt door de gezondheid van de kat en de verschillende types voeding die hij krijgt (**Chapter 3**).

De darmmicrobiota leeft van wat er overblijft na enzymatische vertering in de gastheer. Die onverteerde restfracties zijn sterk verschillend tussen huiskatten, die vooral commerciële voeders (met plantaardige ingrediënten) krijgen, of grote exotische katachtigen, die een breed scala aan prooien verorberen, al dan niet met weinig enzymatisch verteerbare dierlijke weefsels zoals huid, bindweefsel, veren en bot. Nutritionele strategieën die de gezondheid en het welzijn van katachtigen in gevangenschap bevorderen vormen een belangrijk onderdeel van een uitgekiende *ex situ* conservatiestrategie. Een aspect dat hierbij echter uit het oog wordt verloren is de impact van de verschillende types dierlijke componenten op de balans tussen de gastheer en zijn gastro-intestinale microbiële gemeenschap. Deze laatste is echter nog zo goed als ongekend in de meeste grote exotische katachtigen.

In dit proefschrift wordt voor het eerst de diversiteit van de gastro-intestinale microbiële gemeenschap van cheeta's in gevangenschap beschreven. Hiervoor werden faecale stalen van 50 volwassen cheeta's uit 13 verschillende zoos van 8 Europese landen onderworpen aan een set van cultuur-onafhankelijke technieken (**Chapters 4, 6 & 7**). Hieruit blijkt dat bacteriële groepen behorende tot het phylum Firmicutes zowel in hoeveelheid als verscheidenheid alle anderen overtreffen, gevolgd door een kleinere hoeveelheid Actinobacteria, Proteobacteria en Fusobacteria. Binnen de Firmicutes zijn *Clostridium* clusters XIVa, XI en I de hoofdrolspelers. Deze resultaten werden gespiegeld tegen microbiële data, verkregen voor een kleiner aantal in het wild levende cheeta's uit Namibië. Ondanks het grote aantal omgevingsgebonden factoren dat beide populaties onderscheidt, worden de in het wild levende cheeta's ook vergezeld van dezelfde hoofdrolspelers in een even rijkelijke faecale microbiota. Proportionele verschillen tussen bacteriële groepen houden mogelijks verband met verschillen in dieettype en eetgedrag en het hieruit resulterend verschillend aanbod van de onverteerde restfractie besschikbaar voor de microbiota. In vergelijking met data verkregen voor een aantal huiskatten blijken in deze laatste een hele reeks andere bacteriële groepen de hoofdrol te spelen. Deze wezenlijke verschillen tussen beide species ondersteunen de these dat de verschillende eetpatronen in katachtigen misschien wel grotere sporen achterlaten in de samenstelling van hun darmmicrobiota dan aanvankelijk gedacht.

In dit proefschrift staan ook de resultaten van de eerste langdurige longitudinale studie waarin de dynamiek van de faecale microbiota in een strikte carnivoor wordt opgevolgd (**Chapter 5**). Een tweemaandelijkse staalname in vijf dieren uit twee Europese zoos gedurende drie jaar resulteert in een schat van data die aangeeft dat de samenstelling van de dominante bacteriële groepen stabiel is, maar een periode van ziekte deze stabiliteit grondig kan verstoren.

Ten slotte wordt in dit proefschrift de Simulator van het Humaan Intestinaal Microbieel Ecosysteem (SHIME) omgetoverd tot een simulator van het gastro-intestinaal microbieel ecosysteem in de cheeta (**Chapter 8**). Model parameters en nutritionele condities (*i.e.* rijk aan eiwitten, arm aan koolhydraten) voor dit dynamisch *in vitro* model werden zodanig gekozen dat de *in vivo* condities in de darm van deze

strikte carnivoor zo goed mogelijk werden nabootst. Na inoculatie van het model met faecale microbiota ontstond na ongeveer twee weken een microbiële en metabole stabiliteit die nog gedurende één maand kon worden aangehouden. Na de initiële stabilisatie werd dit dynamisch *in vitro* model geëvalueerd als tool voor nutritionele sturing van het metabolisme van de microbiota door het inbrengen van collageen als testsubstraat. Hierbij werd ook de toepassing van metabolomics voor fermentatiestalen gedetailleerder onder de loep genomen. Deze doorgedreven screening van metabolieten, het ultieme eindresultaat van microbieel metabolisme, heeft het potentieel om de crosstalk tussen bacteriën enerzijds en tussen gastheer en bacterie anderzijds verder uit te diepen.

Uiteindelijk willen we nog dit meegeven: het in kaart brengen van de microbiële gemeenschap verscholen in het spijsverteringskanaal van cheeta's in gevangenschap effent het pad om verder de complexe, maar uiterst fascinerende samenwerking tussen deze carnivore gastheer en zijn microbiële gezellen te ontrafelen. Iets dat deze 'hazewind van de woestijn' en bij uitbreiding ook andere kwetsbare en bedreigde diersoorten alleen maar ten goede kan komen.

Bibliography

1. Agnarsson I, Kuntner M, May-Collado LJ (2010) Dogs, cats, and kin: a molecular species-level phylogeny of Carnivora. Mol Phylogenet Evol 54: 726–745.

2. Van Valkenburgh B (2007) Deja vu: the evolution of feeding morphologies in the Carnivora. Integr Comp Biol 47: 147–163.

3. Lander E, Linton L, Birren B, Nusbaum C, Zody M, et al. (2001) Initial sequencing and analysis of the human genome. Nature 409: 860–921.

4. Montague MJ, Li G, Gandolfi B, Khan R, Aken BL, et al. (2014) Comparative analysis of the domestic cat genome reveals genetic signatures underlying feline biology and domestication. Proc Natl Acad Sci 111: 17230–17235.

5. Pontius JU, Mullikin JC, Smith DR, Team AS, Lindblad-toh K, et al. (2007) Initial sequence and comparative analysis of the cat genome: 1675–1689.

6. O'Brien SJ, Johnson W, Driscoll C, Pontius J, Pecon-Slattery J, et al. (2008) State of cat genomics. Trends Genet 24: 268–279.

7. Krausman PR, Morales SM (2005) Acinonyx jubatus. Mamm Species 771: 1–6.

8. O'Brien SJ, Wildt DE, Bush M, Caro TM, Fitzgibbon C, et al. (1987) East African cheetahs: Evidence for two population bottlenecks? Proc Natl Acad Sci 84: 508–511.

9. Zordan M, Deem SL, Sanchez CR (2012) Focal palatine erosion in captive and free-living cheetahs (Acinonyx jubatus) and other felid species. Zoo Biol 31: 181–188.

10. O'Brien SJ, Johnson W (2005) Big Cat Genomics. Annu Rev Genomics Hum Genet 6: 407–429.

11. Marker LL, Dickman AJ (2003) Morphology, physical condition, and growth of the Cheetah (Acinonyx jubatus jubatus). J Mammal 84: 840–850.

12. O'Brien SJ, Roelke ME, Marker L, Newman A, Winkler CA, et al. (1985) Genetic basis for species vulnerability in the cheetah. Science (80-) 227: 1428–1434.

13. Castro-Prieto A, Wachter B, Sommer S (2011) Cheetah paradigm revisited: MHC diversity in the world's largest free-ranging population. Mol Biol Evol 28: 1455–1468.

14. Weber D, Stewart B, Schienman J, Lehman N (2004) Major histocompatibility complex variation at three class II loci in the northern elephant seal. Mol Ecol 13: 711–718.

15. Merola M (1994) A reassessment of homozygosity and the case for inbreeding depression in the Cheetah, Acinonyx jubatus: Implications for conservation. Conserv Bioogy 8: 961–971.

16. Caro TM, Laurenson MK (1994) Ecological and genetic factors in conservation: a cautionary tale. Science (80-) 263: 485–486.

17. Ellegren H, Hartman G, Johansson M, Andersson L (1993) Major histocompatibility complex monomorphism and low levels of DNA fingerprinting variability in a reintroduced and rapidly expanding population of beavers. Proc Natl Acad Sci 90: 8150–8153.

18. Charruau P, Fernandes C, Orozco-Terwengel P, Peters J, Hunter L, et al. (2011) Phylogeography, genetic structure and population divergence time of cheetahs in Africa and Asia: evidence for long-term geographic isolates. Mol Ecol 20: 706–724.

19. Bell KM (2010) Spot the difference: are cheetas really just big cats? Nottingham, UK: Nottingham University Press.

20. Christiansen P, Wroe S (2007) Bite forces and evolutionary adaptations to feeding ecology in carnivores. Ecology 88: 347–358.

21. Ripple WJ, Estes JA, Beschta RL, Wilmers CC, Ritchie EG, et al. (2014) Status and Ecological Effects of the World's Largest Carnivores. Science (80-) 343: 1241484.

22. Ray JC, Hunder L, Zigouris J (2005) Setting conservation and research pririties for larger African carnivores. WCS Working Paper. New York: Wildlife Conservation Society, Vol. 24. pp. 1–203.

23. CITES (2015) Appendices I, II and III: 46.

24. Marker L (2015) International Cheetah Studbook 2014.

25. EAZA (n.d.) http://www.eaza.net/conservation/programmes/.

26. AZA (n.d.) https://www.aza.org/species-survival-plan-program/.

27. Crosier AE, Marker L, Howard J, Pukazhenthi BS, Henghali JN, et al. (2007) Ejaculate traits in the Namibian cheetah (Acinonyx jubatus): influence of age, season and captivity. Reprod Fertil Dev 19: 370.

28. Brown JL (2011) Female reproductive cycles of wild female felids. Anim Reprod Sci 124: 155–162.

29. Wielebnowski NC, Ziegler K, Wildt DE, Lukas J, Brown JL (2002) Impact of social management on reproductive, adrenal and behavioural activity in the cheetah (Acinonyx jubatus). Anim Conserv 5: 291–301.

30. Wielebnowski NC (1999) Behavioral differences as predictors of breeding status in captive cheetahs. Zoo Biol 18: 335–349.

31. Bell KM, Rutherfurd SM, Hendriks WH (2010) Exposure of growing and adult captive cheetahs (Acinonyx jubatus) to dietary isoflavones: twenty years later. J Anim Physiol Anim Nutr (Berl) 94: e329–38.

32. Munson L, Terio KA, Worley M, Jago M, Bagot-Smith A, et al. (2005) Extrinsic factors significantly affect patterns of disease in free-ranging and captive cheetah (Acinonyx jubatus) populations. J Wildl Dis 41: 542–548.

33. Koester DC, Freeman EW, Brown JL, Wildt DE, Terrell K a, et al. (2015) Motile Sperm Output by Male Cheetahs (Acinonyx jubatus) Managed Ex Situ Is Influenced by Public Exposure and Number of Care-Givers. PLoS One 10: e0135847.

34. Kotsch V, Kubber-Heiss A, Url A, Walzer C, Schmidt R (2002) Diseases of captive cheetahs (Acinonyx jubatus) within the European Endangered Species Program (EEP) - a 22-year retrospective histopathological study. Wien Tierarztl Monatsschr 89: 341–350.

35. Gosselin SJ, Loudy DL, Tarr MJ, Balistreri WF, Setchell KDR, et al. (1988) Veno-occlusive disease of the liver in captive cheetah. Vet Pathol 25: 48–57.

36. Terio KA, Marker L, Munson L (2004) Evidence for chronic stress in captive but not free-ranging cheetahs (Acinonyx jubatus) based on adrenal morphology and function. J Wildl Dis 40: 259–266.

37. Terio KA, Munson L, Moore PF (2012) Characterization of the gastric immune response in cheetahs (Acinonyx jubatus) with Helicobacter-associated gastritis. Vet Pathol 49: 824–833.

38. Lane EP, Miller S, Lobetti R, Caldwell P, Bertschinger HJ, et al. (2011) Effect of diet on the incidence of and mortality owing to gastritis and renal disease in captive cheetahs (Acinonyx jubatus) in South Africa. Zoo Biol 14: 1–14.

39. Whitehouse-Tedd KM, Lefebvre SL, Janssens GPJ (2015) Dietary factors associated with faecal consistency and other indicators of gastrointestinal health in the captive cheetah (Acinonyx jubatus). PLoS One 10: e0120903.

40. Allan G, Portas T, Bryant B, Howlett R, Blyde D (2008) Ulnar metaphyseal osteochondrosis in seven captive bred cheetahs (Acinonyx Jubatus). Vet Radiol Ultrasound 49: 551–556.

41. Ziegler-Meeks K (2009) Husbandry Manual for the Cheetah. White Oak Conservation Center.

42. Bond JC, Lindburg DG (1990) Carcass feeding of captive cheetahs (Acinonyx jubatus): the effects of a naturalistic feeding program on oral health and psychological well-being. Appl Anim Behav Sci 26: 373–382.

43. Venter EH, van Vuuren M, Carstens J, Walt ML Van Der, Nieuwoudt B, et al. (2003) A molecular epidemiologic investigation of Salmonella from a meat source to the feces of captive cheetah (Acinonyx jubatus). J Zoo Wildl Med 34: 76–81.

44. Robert N, Walzer C (2009) Pathological disorders in captive cheetahs. Iberian Lynx Ex Situ Conservation: an interdisciplinary approach. pp. 265–272.

45. Citino SB (1994) Diagnosis of Clostridium perfringens enterotoxicosis in a collection of cheetahs (Acinonyx jubatus jubatus). Proceedings of the Association of Reptile Amphibian Veterinarians. pp. 301–302.

46. Van Valkenburgh B (1996) Feeding behavior in free-ranging, large African carnivores. J Mammal 77: 240–254.

47. Dierenfeld ES (1996) Nutritional Wisdom: Adding the Science to the art. Zoo Biol 15: 447–448.

48. Bechert U, Mortenson J, Dierenfeld ES, Cheeke P, Keller M, et al. (2002) Diet composition and blood values of captive cheetahs (Acinonyx jubatus) fed either supplemented meat or commercial food preparations. J Zoo Wildl Med 33: 16–28.

49. Depauw S, Hesta M, Whitehouse-Tedd K, Vanhaecke L, Verbrugghe A, et al. (2011) Animal fibre: The forgotten nutrient in strict carnivores? First insights in the cheetah. J Anim Physiol Anim Nutr (Berl) 97: 146–154.

50. Mcphee ME (2002) Intact carcasses as enrichment for large felids: Effects on on- and off-exhibit behaviors. Zoo Biol 21: 37–47.

51. Rochus K, Janssens GPJ, Hesta M (2014) Dietary fibre and the importance of the gut microbiota in feline nutrition: a review. Nutr Res Rev 27: 295–307.

52. Depauw S, Bosch G, Hesta M, Whitehouse-Tedd K, Hendriks WH, et al. (2012) Fermentation of animal components in strict carnivores: a comparative study with cheetah fecal inoculum. J Anim Sci 90: 2540–2548.

53. Depauw S, Heilmann RM, Whitehouse-tedd K, Hesta M, Steiner JM, et al. (2014) Effect of diet type on serum and faecal concentration of S100 / calgranulins in the captive cheetah. J Zoo Aquarium Res 2: 33–38.

54. Van Leeuwenhoek A (1684) About animals in the scrurf of the teeth. Philos Trans R Soc London 14: 568–574.

55. Relman D (2008) "Til death do us part": coming to terms with symbiotic relationships. Nat Rev Microbiol 6: 721–724.

56. Aagaard K, Ma J, Antony KM, Ganu R, Petrosino J, et al. (2014) The placenta harbors a unique microbiome. Sci Transl Med 6: 237ra65.

57. Voreades N, Kozil A, Weir TL (2014) Diet and the development of the human intestinal microbiome. Front Microbiol 5: 494.

58. Ottman N, Smidt H, de Vos WM, Belzer C (2012) The function of our microbiota: who is out there and what do they do? Front Cell Infect Microbiol 2: 104.

59. Koenig JE, Spor A, Scalfone N, Fricker AD, Stombaugh J, et al. (2011) Succession of microbial consortia in the developing infant gut microbiome. Proc Natl Acad Sci U S A 108 Suppl : 4578–4585.

60. Dominguez-Bello MG, Costello EK, Contreras M, Magris M, Hidalgo G, et al. (2010) Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proc Natl Acad Sci U S A 107: 11971–11975.

61. Bager P, Wohlfahrt J, Westergaard T (2008) Caesarean delivery and risk of atopy and allergic disease: meta-analyses. Clin Exp Allergy 38: 634–642.

62. Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, et al. (2012) Human gut microbiome viewed across age and geography. Nature 486: 222–227.

63. Schmidt B, Mulder IE, Musk CC, Aminov RI, Lewis M, et al. (2011) Establishment of normal gut microbiota is compromised under excessive hygiene conditions. PLoS One 6: e28284.

64. Arrieta M-C, Stiemsma LT, Amenyogbe N, Brown EM, Finlay B (2014) The intestinal microbiome in early life: health and disease. Front Immunol 5: 427.

65. Penders J, Thijs C, Vink C, Stelma F, Snijders B, et al. (2006) Factors influencing the composition of the intestinal microbiota in early infancy. Pediatrics 118: 511–521.

66. Favier CF, Vaughan EE, De Vos WM, Akkermans a. DL (2002) Molecular monitoring of succession of bacterial communities in human neonates. Appl Environ Microbiol 68: 219–226.

67. Wopereis H, Oozeer R, Knipping K, Belzer C, Knol J (2014) The first thousand days - intestinal microbiology of early life: establishing a symbiosis. Pediatr Allergy Immunol 25: 428–438.

68. El Aidy S, van den Abbeele P, Van de Wiele T, Louis P, Kleerebezem M (2013) Intestinal colonization: how key microbial players become established in this dynamic process: microbial metabolic activities and the interplay between the host and microbes. Bioessays 35: 913–923.

69. Dethlefsen L, Eckburg PB, Bik EM, Relman DA (2006) Assembly of the human intestinal microbiota. Trends Ecol Evol 21: 517–523.

70. Bauer E, Williams B, Smidt H, Mosenthin R, Verstegen M (2006) Influence of dietary components on development of the microbiota in single-stomached species. Nutr Res Rev 19: 63–78.

71. Konstantinov SR, Awati AA, Williams BA, Miller BG, Jones P, et al. (2006) Post-natal development of the porcine microbiota composition and activities. Environ Microbiol 8: 1191–1199.

72. Konstantinov SR, Favier CF, Zhu WY, Williams BA, KlüB J, et al. (2004) Microbial diversity studies of the porcine gastrointestinal ecosystem during weaning transition. Anim Res 53: 317–324.

73. Schokker D, Zhang J, Zhang L-L, Vastenhouw S a, Heilig HGHJ, et al. (2014) Early-life environmental variation affects intestinal microbiota and immune development in new-born piglets. PLoS One 9: e100040.

74. Klein-Jöbstl D, Schornsteiner E, Mann E, Wagner M, Drillich M, et al. (2014) Pyrosequencing reveals diverse fecal microbiota in Simmental calves during early development. Front Microbiol 5: 622.

75. Oikonomou G, Teixeira AGV, Foditsch C, Bicalho ML, Machado VS, et al. (2013) Fecal microbial diversity in pre-weaned dairy calves as described by pyrosequencing of metagenomic 16S rDNA. Associations of Faecalibacterium species with health and growth. PLoS One 8: e63157.

76. Jami E, Israel A, Kotser A, Mizrahi I (2013) Exploring the bovine rumen bacterial community from birth to adulthood. ISME J 7: 1069–1079.

77. Combes S, Michelland RJ, Monteils V, Cauquil L, Soulié V, et al. (2011) Postnatal development of the rabbit caecal microbiota composition and activity. FEMS Microbiol Ecol 77: 680–689.

78. Earing JE, Durig AC, Gellin GL, Lawrence LM, Flythe MD (2012) Bacterial colonization of the equine gut; comparison of mare and foal pairs by PCR-DGGE. Adv Microbiol 2: 79–86.

79. Stevenson TJ, Buck CL, Duddleston KN (2014) Temporal dynamics of the cecal gut microbiota of juvenile arctic ground squirrels: a strong litter effect across the first active season. Appl Environ Microbiol 80: 4260–4268.

80. Xue Z, Zhang W, Wang L, Hou R, Zhang M, et al. (2015) The bamboo-eating giant panda harbors a carnivore-like gut microbiota, with excessive seasonal variations. MBio 6: e00022–15.

81. Ardeshir A, Narayan NR, Méndez-lagares G, Lu D, Rauch M, et al. (2014) Breast-fed and bottle-fed infant rhesus macaques develop distinct gut microbiotas and immune systems. Sci Transl Med 6: 252ra120.

82. Sullivan AO, He X, Mcniven EMS, Haggarty NW, Lo B, et al. (2013) Early diet impacts infant rhesus gut microbiome, immunity, and metabolism. J Proteome Res 12: 2833–2845.

83. Jia J, Frantz N, Khoo C, Gibson GR, Rastall RA, et al. (2011) Investigation of the faecal microbiota of kittens: monitoring bacterial succession and effect of diet. FEMS Microbiol Ecol 78: 395–404.

84. Buddington RK (2003) Postnatal changes in bacterial populations in the gastrointestinal tract of dogs. Am J Vet Res 64: 646–651.

85. Vester B, Dalsing B, Middelbos I, Apanavicius C, Lubbs D, et al. (2009) Faecal microbial populations of growing kittens fed high- or moderate-protein diets. Arch Anim Nutr 63: 254–265.

86. Ley RE, Hamady M, Lozupone C, Turnbaugh PJ, Ramey RR, et al. (2008) Evolution of mammals and their gut microbes. Science (80-) 320: 1647–1651.

87. Faith JJ, Guruge JL, Charbonneau M, Subramanian S, Seedorf H, et al. (2013) The long-term stability of the human gut microbiota. Science 341: 1237439.

88. Lozupone CA, Stombaugh JI, Gordon JI, Jansson JK, Knight R (2012) Diversity, stability and resilience of the human gut microbiota. Nature 489: 220–230.

89. Caporaso JG, Lauber CL, Costello EK, Berg-Lyons D, Gonzalez A, et al. (2011) Moving pictures of the human microbiome. Genome Biol 12: R50.

90. Bäckhed F, Fraser CM, Ringel Y, Sanders ME, Sartor RB, et al. (2012) Defining a healthy human gut microbiome: current concepts, future directions, and clinical applications. Cell Host Microbe 12: 611–622.

91. Doré J, Corthier G (2010) The human intestinal microbiota. Gastroentérologie Clin Biol 34: S7–15.

92. Lozupone CA, Stombaugh J, Gonzalez A, Ackermann G, Jansson JK, et al. (2013) Meta-analyses of studies of the human microbiota. Genome Res 23: 1704–1714.

93. Tap J, Mondot S, Levenez F, Pelletier E, Caron C, et al. (2009) Towards the human intestinal microbiota phylogenetic core. Environ Microbiol 11: 2574–2584.

94. Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, et al. (2011) Enterotypes of the human gut microbiome. Nature 473: 174–180.

95. Wu GD, Chen J, Hoffmann C, Bittinger K, Chen Y-Y, et al. (2011) Linking long-term dietary patterns with gut microbial enterotypes. Science 334: 105–108.

96. Blumberg R, Powrie F (2012) Microbiota, disease, and back to health: a metastable journey. Sci Transl Med 4: 137rv7.

97. Kinross JM, Darzi AW, Nicholson JK (2011) Gut microbiome-host interactions in health and disease. Genome Med 3: 14.

98. Sekirov I, Russell SL, Antunes LCM, Finlay BB (2010) Gut Microbiota in Health and Disease. Physiol Rev 90: 859–904.

99. Korpela K, Flint HJ, Johnstone AM, Lappi J, Poutanen K, et al. (2014) Gut microbiota signatures predict host and microbiota responses to dietary interventions in obese individuals. PLoS One 9: e90702.

100. Nicholson JK, Holmes E, Kinross J, Burcelin R, Gibson G, et al. (2012) Host-gut microbiota metabolic interactions. Science 336: 1262–1267.

101. Holmes E, Kinross J, Gibson GR, Burcelin R, Jia W, et al. (2012) Therapeutic modulation of microbiota-host metabolic interactions. Sci Transl Med 4: 137rv6.

102. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, et al. (2010) A human gut microbial gene catalogue established by metagenomic sequencing. Nature 464: 59–65.

103. Janeway C, Travers P, Walport M, Shlomchik M (2001) Immunobiology. 5th ed. New York: Garland Science.

104. Belkaid Y, Hand TW (2014) Review Role of the Microbiota in Immunity and Inflammation. Cell 157: 121–141.

105. Cerf-Bensussan N, Gaboriau-Routhiau V (2010) The immune system and the gut microbiota: friends or foes? Nat Rev Immunol 10: 735–744.

106. Bauer E, Williams BA, Verstegen MWA (2006) Influence of the Gastrointestinal Microbiota on Development of the Immune System in Young Animals. Curr Issues Intest Microbiol 7: 35–52.

107. Macpherson AJ, Harris NL (2004) Interactions between commensal intestinal bacteria and the immune system. Nat Rev Immunol 4: 478–485.

108. Stappenbeck TS, Hooper L V, Gordon JI (2002) Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells. PNAS 99: 15451–15455.

109. Macpherson AJ, Hunziker L, McCoy K, Lamarre A (2001) IgA responses in the intestinal mucosa against pathogenic and non-pathogenic microorganisms. Microbes Infect 3: 1021–1035.

110. Buffie CG, Pamer EG (2013) Microbiota-mediated colonization resistance against intestinal pathogens. Nat Rev Immunol 13: 790–801.

111. Dinan TG, Stilling RM, Stanton C, Cryan JF (2015) Collective unconscious: How gut microbes shape human behavior. J Psychiatr Res 63: 1–9.

112. McVey Neufeld KA, Mao YK, Bienenstock J, Foster JA, Kunze WA (2013) The microbiome is essential for normal gut intrinsic primary afferent neuron excitability in the mouse. Neurogastroenterol Motil 25: 183–e88.

113. Neufeld KM, Kang N, Bienenstock J, Foster J a (2011) Reduced anxiety-like behavior and central neurochemical change in germ-free mice. Neurogastroenterol Motil 23: e119.

114. Bercik P, Denou E, Collins J, Jackson W, Lu J, et al. (2011) The intestinal microbiota affect central levels of brain-derived neurotropic factor and behavior in mice. Gastroenterology 141: 599–609.

115. Krajmalnik-brown R, Lozupone C, Kang D, Adams JB (2015) Gut bacteria in children with autism spectrum disorders: challenges and promise of studying how a complex community influences a complex disease. Microb Ecol Health Dis 26: 26914.

116. Lombardo MP (2008) Access to mutualistic endosymbiotic microbes: an underappreciated benefit of group living. Behav Ecol Sociobiol 62: 479–497.

117. Troyer K (1984) Microbes , herbivory and the evolution social behavior. J Theor Biol 106: 157–169.

118. Montiel-Castro AJ, González-Cervantes RM, Bravo-Ruiseco G, Pacheco-López G (2013) The microbiota-gut-brain axis: neurobehavioral correlates, health and sociality. Front Integr Neurosci 7: 70.

119. O'Mahony SM, Hyland NP, Dinan TG, Cryan JF (2011) Maternal separation as a model of brain-gut axis dysfunction. Psychopharmacology (Berl) 214: 71–88.

120. Desbonnet L, Garrett L, Clarke G, Kiely B, Cryan JF, et al. (2010) Effects of the probiotic Bifidobacterium infantis in the maternal separation model of depression. Neuroscience 170: 1179–1188.

121. Messaoudi M, Violle N, Bisson J-F, Desor D, Javelot H, et al. (2011) Beneficial psychological effects of a probiotic formulation (Lactobacillus helveticus R0052 and Bifidobacterium longum R0175) in healthy human volunteers. Gut Microbes 2: 256–261.

122. Sousa T, Paterson R, Moore V, Carlsson A, Abrahamsson B, et al. (2008) The gastrointestinal microbiota as a site for the biotransformation of drugs. Int J Pharm 363: 1–25.

123. Carmody RN, Turnbaugh PJ (2014) Host-microbial interactions in the metabolism of therapeutic and diet-derived xenobiotics. J Clin Invest 124: 4173–4181.

124. Okuda H, Ogura K, Kato A, Takubo H, Watabe T (1998) A possible mechanism of eighteen patient deaths caused by interactions of sorivudine , a new antiviral drug , with oral 5-fluorouracil prodrugs. J Pharmacol Exp Ther 287: 791–799.

125. Kim D-H (2015) Gut microbiota-mediated drug-antibiotic interactions. Drug Metab Dispos 43: 1581–1589.

126. De Godoy MRC, Kerr KR, Fahey GC (2013) Alternative dietary fiber sources in companion animal nutrition. Nutrients 5: 3099–3117.

127. Peachey SE, Dawson JM, Harper EJ (2000) Gastrointestinal transit times in young and old cats. Comp Biochem Physiol A Mol Integr Physiol 126: 85–90.

128. Jacobs D, Gaudier E, Duynhoven J, Vaughan E (2009) Non-digestible food ingredients, colonic microbiota and the impact on gut health and immunity: a role for metabolomics. Curr Drug Metab 10: 41–54.

129. Jansson J, Willing B, Lucio M, Fekete A, Dicksved J, et al. (2009) Metabolomics reveals metabolic biomarkers of Crohn's disease. PLoS One 4: e6386.

130. Martínez I, Kim J, Duffy PR, Schlegel VL, Walter J (2010) Resistant starches types 2 and 4 have differential effects on the composition of the fecal microbiota in human subjects. PLoS One 5: e15046.

131. Jones JM (2014) CODEX-aligned dietary fiber definitions help to bridge the "fiber gap". Nutr J 13: 34.

132. Standards IF (2015) Codex Alimentarius: Guidelines on nutrition labelling CAC/GL2-1985.

133. Flint HJ, Scott K, Duncan SH (2012) Microbial degradation of complex carbohydrates in the gut. Gut Microbes 3: 289–306.

134. Bergman EN (1990) Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiol Rev 70: 567–590.

135. Topping DL, Clifton PM (2001) Short-chain fatty qcids and human colonic function: roles of resistant starch and nonstarch polysaccharides. Physiol Rev 81: 1031–1064.

136. Wong JMW, de Souza R, Kendall CWC, Emam A, Jenkins DJ a. (2006) Colonic Health: fermentation and short chain fatty acids. J Clin Gastroenterol 40: 235–243.

137. Louis P, Scott KP, Duncan SH, Flint HJ (2007) Understanding the effects of diet on bacterial metabolism in the large intestine. J Appl Microbiol 102: 1197–1208.

138. Belenguer A, Duncan SH, Calder AG, Holtrop G, Louis P, et al. (2006) Two routes of metabolic cross-feeding between Bifidobacterium adolescentis and butyrate-producing anaerobes from the human gut. Appl Environ Microbiol 72: 3593–3599.

139. Lee W-J, Hase K (2014) Gut microbiota-generated metabolites in animal health and disease. Nat Chem Biol 10: 416–424.

140. Hosseini E, Grootaert C, Verstraete W, Van de Wiele T (2011) Propionate as a health-promoting microbial metabolite in the human gut. Nutr Rev 69: 245–258.

141. Hamer HM, Jonkers D, Venema K, Vanhoutvin S, Troost FJ, et al. (2008) Review article: the role of butyrate on colonic function. Aliment Pharmacol Ther 27: 104–119.

142. Windey K, De Preter V, Verbeke K (2012) Relevance of protein fermentation to gut health. Mol Nutr Food Res 56: 184–196.

143. Demeyer D, Mertens B, De Smet S, Ulens M (2015) Mechanisms linking colorectal cancer to the consumption of (processed) red meat: a review. Crit Rev Food Sci Nutr.

144. Alexander DD, Cushing CA, Lowe KA, Sceurman B, Roberts MA (2009) Meta-analysis of animal fat or animal protein intake and colorectal cancer. Am J Clin Nutr 89: 1402–1409.

145. Bouvard V, Loomis D, Guyton KZ, Grosse Y, Ghissassi F El, et al. (2015) Carcinogenicity of consumption of red and processed meat. Lancet Oncol 16: 1599–1600.

146. Blachier F, Mariotti F, Huneau JF, Tomé D (2007) Effects of amino acid-derived luminal metabolites on the colonic epithelium and physiopathological consequences. Amino Acids 33: 547–562.

147. Derrien M, Van Baarlen P, Hooiveld G, Norin E, Müller M, et al. (2011) Modulation of mucosal immune response, tolerance, and proliferation in mice colonized by the mucin-degrader Akkermansia muciniphila. Front Microbiol 2: 166.

148. LeBlanc JG, Milani C, de Giori GS, Sesma F, van Sinderen D, et al. (2013) Bacteria as vitamin suppliers to their host: a gut microbiota perspective. Curr Opin Biotechnol 24: 160–168.

149. Wilson E (1994) Naturalist. New York: Warner Books.

150. Hurlbert S (1971) The nonconcept of species diversity: a critique and alternative paramete. Ecology 52: 577–586.

151. Eberhardt L (1969) Some aspects of species diversity models. Ecology 50: 503–505.

152. Dunlap P V (2001) Microbial Diversity. Encycl Biodivers 4: 191–205.

153. Bent SJ, Forney LJ (2008) The tragedy of the uncommon: understanding limitations in the analysis of microbial diversity. ISME J 2: 689–695.

154. Whittaker R (1972) Evolution and measurement of species diversity. Taxon 21: 213–251.

155. Peet RK (1974) The measurement of species diversity. Annu Rev Ecol Syst 5: 285–307.

156. Chiarucci A, Bacaro G, Scheiner SM (2011) Old and new challenges in using species diversity for assessing biodiversity. Philos Trans R Soc Lond B Biol Sci 366: 2426–2437.

157. Lozupone CA, Knight R (2008) Species divergence and the measurement of microbial diversity. FEMS Microbiol Rev 32: 557–578.

158. Curtis TP, Sloan WT, Scannell JW (2002) Estimating prokaryotic diversity and its limits. PNAS 99: 10494–10499.

159. Hill TCJ, Walsh KA, Harris JA, Moffett BF (2003) Using ecological diversity measures with bacterial communities. FEMS Microbiol Ecol 43: 1–11.

160. Mayr E (1942) Systematics and the origin of species from the viewpoint of a zoologist. New York: Colombia University Press.

161. Achtman M, Wagner M (2008) Microbial diversity and the genetic nature of microbial species. Nat Rev Microbiol 6: 431–440.

162. Gevers D, Cohan FM, Lawrence JG, Spratt BG, Coenye T, et al. (2005) Re-evaluating prokaryotic species. Nat Rev Microbiol 3: 733–739.

163. Kim M, Oh H-S, Park S-C, Chun J (2014) Towards a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of prokaryotes. Int J Syst Evol Microbiol 64: 346–351.

164. Goris J, Konstantinidis KT, Klappenbach JA, Coenye T, Vandamme P, et al. (2007) DNA-DNA hybridization values and their relationship to whole-genome sequence similarities. Int J Syst Evol Microbiol 57: 81–91.

165. Rosselló-Móra R, Amann R (2015) Past and future species definitions for Bacteria and Archaea. Syst Appl Microbiol 38: 209–216.

166. Richter M, Rosselló-Móra R (2009) Shifting the genomic gold standard for the prokaryotic species definition. Proc Natl Acad Sci U S A 106: 19126–19131.

167. Kämpfer P, Rossello-Mora R (2004) The species concept for prokaryotic microorganisms - An obstacle for describing diversity? Poiesis Prax 3: 62–72.

168. Rosselló-Mora R, Amann R (2001) The species concept for prokaryotes. FEMS Microbiol Rev 25: 39–67.

169. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Sargent M, et al. (2005) Diversity of the human intestinal microbial flora. Science (80-) 308: 1635–1638.

170. Huse SM, Young VB, Morrison HG, Antonopoulos D a, Kwon J, et al. (2014) Comparison of brush and biopsy sampling methods of the ileal pouch for assessment of mucosa-associated microbiota of human subjects. Microbiome 2: 5.

171. Durbán A, Abellán JJ, Jiménez-Hernández N, Ponce M, Ponce J, et al. (2011) Assessing gut microbial diversity from feces and rectal mucosa. Microb Ecol 61: 123–133.

172. Hamady M, Knight R (2009) Microbial community profiling for human microbiome projects : Tools, techniques, and challenges. Genome Res 19: 1141–1152.

173. Tedjo DI, Jonkers DMAE, Savelkoul PH, Masclee AA, van Best N, et al. (2015) The effect of sampling and storage on the fecal microbiota composition in healthy and diseased subjects. PLoS One 10: e0126685.

174. Ott SJ, Musfeldt M, Timmis KN, Hampe J, Wenderoth DF, et al. (2004) In vitro alterations of intestinal bacterial microbiota in fecal samples during storage. Diagn Microbiol Infect Dis 50: 237–245.

175. Lauber CL, Zhou N, Gordon JI, Knight R, Fierer N (2010) Effect of storage conditions on the assessment of bacterial community structure in soil and human-associated samples. FEMS Microbiol Lett 307: 80–86.

176. Bahl MI, Bergström A, Licht TR (2012) Freezing fecal samples prior to DNA extraction affects the Firmicutes to Bacteroidetes ratio determined by downstream quantitative PCR analysis. FEMS Microbiol Lett 329: 193–197.

177. Wagner Mackenzie B, Waite DW, Taylor MW (2015) Evaluating variation in human gut microbiota profiles due to DNA extraction method and inter-subject differences. Front Microbiol 6: 130.

178. Kennedy NA, Walker AW, Berry SH, Duncan SH, Farquarson FM, et al. (2014) The impact of different DNA extraction kits and laboratories upon the assessment of human gut microbiota composition by 16S rRNA gene sequencing. PLoS One 9: e88982.

179. Yu Z, Morrison M (2004) Improved extraction of PCR-quality community DNA from digesta and fecal samples. Biotechniques 36: 808–812.

180. McOrist AL, Jackson M, Bird AR (2002) A comparison of five methods for extraction of bacterial DNA from human faecal samples. J Microbiol Methods 50: 131–139.

181. Maukonen J, Simões C, Saarela M (2012) The currently used commercial DNA-extraction methods give different results of clostridial and actinobacterial populations derived from human fecal samples. FEMS Microbiol Ecol 79: 697–708.

182. Schrader C, Schielke a, Ellerbroek L, Johne R (2012) PCR inhibitors - occurrence, properties and removal. J Appl Microbiol 113: 1014–1026.

183. Inglis GD, Thomas MC, Thomas DK, Kalmokoff ML, Brooks SPJ, et al. (2012) Molecular methods to measure intestinal bacteria : a review. J AOAC Int 95: 5–24.

184. Kanagawa T (2003) Bias and artifacts in multitemplate polymerase chain reactions (PCR). J Biosci Bioeng 96: 317–323.

185. Kleessen B, Bezirtzoglou E, Mättö J (2000) Culture-based knowledge on biodiversity, development and stability of human gastrointestinal microflora. Microb Ecol Health Dis Suppl 2: 53–63.

186. Kaeberlein T, Lewis K, Epstein SS (2002) Isolating " uncultivable " microorganisms in pure culture in a simulated natural environment. Science (80-) 296: 1127–1130.

187. Rappé MS, Giovannoni SJ (2003) The uncultured microbial majority. Annu Rev Microbiol 57: 369–394.

188. Goodman AL, Kallstrom G, Faith JJ, Reyes A, Moore A, et al. (2011) Extensive personal human gut microbiota culture collections characterized and manipulated in gnotobiotic mice. PNAS 108: 6252–6257.

189. Lagier J-C, Hugon P, Khelaifia S, Fournier P-E, La Scola B, et al. (2015) The rebirth of culture in microbiology through the example of culturomics to study human gut microbiota. Clin Microbiol Rev 28: 237–264.

190. Walker AW, Duncan SH, Louis P, Flint HJ (2014) Phylogeny, culturing, and metagenomics of the human gut microbiota. Trends Microbiol 22: 267–274.

191. Zoetendal EG, Cheng B, Koike S, Mackie RI (2005) Molecular microbial ecology of the gastrointestinal tract : from phylogeny to function. Curr Issues Intest Microbiol 5: 31–48.

192. Namsolleck P, Thiel R, Lawson P, Holmstrøm K, Rajilic M, et al. (2004) Molecular methods for the analysis of gut microbiota. Microb Ecol Health Dis 16: 71–85.

193. Mariat D, Firmesse O, Levenez F, Guimarães V, Sokol H, et al. (2009) The Firmicutes/Bacteroidetes ratio of the human microbiota changes with age. BMC Microbiol 9: 123.

194. Guo X, Xia X, Tang R, Zhou J, Zhao H, et al. (2008) Development of a real-time PCR method for Firmicutes and Bacteroidetes in faeces and its application to quantify intestinal population of obese and lean pigs. Lett Appl Microbiol 47: 367–373.

195. Malinen E, Rinttilä T, Kajander K, Mättö J, Kassinen A, et al. (2005) Analysis of the fecal microbiota of irritable bowel syndrome patients and healthy controls with real-time PCR. Am J Gastroenterol 100: 373–382.

196. Song Y, Liu C, Finegold SM (2004) Real-time PCR quantitation of clostridia in feces of autistic children real-time PCR quantitation of clostridia in Feces of autistic children. Appl Environ Microbiol 70: 6459–6465.

197. Bartosch S, Fite A, Macfarlane GT, McMurdo MET (2004) Characterization of bacterial communities in feces from healthy elderly volunteers and hospitalized elderly patients by using real-time PCR and effects of antibiotic treatment on the fecal microbiota. Appl Environ Microbiol 70: 3575–3581.

198. Smith CJ, Osborn AM (2009) Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology. FEMS Microbiol Ecol 67: 6–20.

199. Sankar SA, Lagier J-C, Pontarotti P, Raoult D, Fournier P-E (2015) The human gut microbiome, a taxonomic conundrum. Syst Appl Microbiol 38: 276–286.

200. Tottey W, Denonfoux J, Jaziri F, Parisot N, Missaoui M, et al. (2013) The human gut chip "HuGChip", an explorative phylogenetic microarray for determining gut microbiome diversity at family level. PLoS One 8: e62544.

201. Rajilić-Stojanović M, Heilig HGHJ, Molenaar D, Kajander K, Surakka A, et al. (2009) Development and application of the human intestinal tract chip, a phylogenetic microarray: analysis of universally conserved phylotypes in the abundant microbiota of young and elderly adults. Environ Microbiol 11: 1736–1751.

202. Paliy O, Kenche H, Abernathy F, Michail S (2009) High-throughput quantitative analysis of the human intestinal microbiota with a phylogenetic microarray. Appl Environ Microbiol 75: 3572–3579.

203. Khoo C, Brockman J, JMBM V, Schuren F, Grosse K (2007) Canine and feline microbiota as analyzed by the human I-chip cluster in different groups. FASEB J 21: 705.16.

204. Budding AE, Grasman ME, Lin F, Bogaards JA, Soeltan-Kaersenhout DJ, et al. (2010) IS-pro: high-throughput molecular fingerprinting of the intestinal microbiota. FASEB J 24: 4556–4564.

205. Joossens M, Huys G, Van Steen K, Cnockaert M, Vermeire S, et al. (2011) High-throughput method for comparative analysis of denaturing gradient gel electrophoresis profiles from human fecal samples reveals significant increases in two bifidobacterial species after inulin-type prebiotic intake. FEMS Microbiol Ecol 75: 343–349.

206. Duytschaever G, Huys G, Bekaert M, Boulanger L, De Boeck K, et al. (2011) Cross-sectional and longitudinal comparisons of the predominant fecal microbiota compositions of a group of pediatric patients with cystic fibrosis and their healthy siblings. Appl Environ Microbiol 77: 8015–8024.

207. Liszt K, Zwielehner J, Handschur M, Hippe B, Thaler R, et al. (2009) Characterization of bacteria, clostridia and Bacteroides in faeces of vegetarians using qPCR and PCR-DGGE fingerprinting. Ann Nutr Metab 54: 253–257.

208. Hayashi H, Takahashi R, Nishi T, Sakamoto M, Benno Y (2005) Molecular analysis of jejunal, ileal, caecal and recto-sigmoidal human colonic microbiota using 16S rRNA gene libraries and terminal restriction fragment length polymorphism. J Med Microbiol 54: 1093–1101.

209. Dimitriu PA, Boyce G, Samarakoon A, Hartmann M, Johnson P, et al. (2013) Temporal stability of the mouse gut microbiota in relation to innate and adaptive immunity. Environ Microbiol Rep 5: 200–210.

210. Vanhoutte T, Huys G, De Brandt E, Swings J (2004) Temporal stability analysis of the microbiota in human feces by denaturing gradient gel electrophoresis using universal and group-specific 16S rRNA gene primers. FEMS Microbiol Ecol 48: 437–446.

211. Suchodolski JS, Ruaux CG, Steiner M, Fetz K, Williams DA (2004) Application of molecular fingerprinting for qualitative assessment of small-intestinal bacterial diversity in dogs. J Clin Microbiol 42: 4702–4708.

212. Huys G, Vanhoutte T, Vandamme P (2008) Application of sequence-dependent electrophoresis fingerprinting in exploring biodiversity and population dynamics of human intestinal microbiota: what can be revealed? Interdiscip Perspect Infect Dis 2008: 597603.

213. Van Dorst J, Bissett A, Palmer AS, Brown M, Snape I, et al. (2014) Community fingerprinting in a sequencing world. FEMS Microbiol Ecol 89: 316–330.

214. Bent SJ, Pierson JD, Forney LJ, Danovaro R, Luna GM, et al. (2007) Measuring species richness based on microbial community fingerprints: the emperor has no clothes. Appl Environ Microbiol 73: 2399–401; author reply 2399–401.

215. Sanger F, Nicklen S, Coulson A (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci 74: 5463–5467.

216. Hayashi H, Sakamoto M, Benno Y (2002) Phylogenetic analysis of the human gut microbiota using 16S rDNA clone libraries and strictly anaerobic culture-based methods. Microbiol Immunol 46: 535–548.

217. De Filippo C, Ramazzotti M, Fontana P, Cavalieri D (2012) Bioinformatic approaches for functional annotation and pathway inference in metagenomics data. Brief Bioinform 13: 696–710.

218. Scholz MB, Lo C-C, Chain PS (2012) Next generation sequencing and bioinformatic bottlenecks: the current state of metagenomic data analysis. Curr Opin Biotechnol 23: 9–15.

219. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, et al. (2010) QIIME allows analysis of high- throughput community sequencing data Intensity normalization improves color calling in SOLiD sequencing. Nat Publ Gr 7: 335–336.

220. Langille MGI, Zaneveld J, Caporaso JG, McDonald D, Knights D, et al. (2013) Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat Biotechnol 31: 814–821.

221. Franzosa EA, Hsu T, Sirota-Madi A, Shafquat A, Abu-Ali G, et al. (2015) Sequencing and beyond: integrating molecular "omics" for microbial community profiling. Nat Rev Microbiol 13: 360–372.

222. Smith MI, Turpin W, Tyler AD, Silverberg MS, Croitoru K (2014) Microbiome analysis - from technical advances to biological relevance. F1000Prime Rep 6: 51.

223. Horvath A, Rozgonyi F, Pesti N, Kocsis E, Malmos G, et al. (2010) Quantitative differences in antibiotic resistance between methicillin-resistant and methicillin-susceptible Staphylococcus aureus strains isolated in Hungary, Austria and Macedonia. J Chemother 22: 246–253.

224. McNulty N, Yatsunenko T, Hsiao A, Faith J, Muegge B., et al. (2011) The impact of a consortium of fermented milk strains on the gut microbiome of gnotobiotic mice and monozygotic twins. Sci Transl Med 3: 106ra106.

225. Booijink CCGM, El-Aidy S, Rajilić-Stojanović M, Heilig HGHJ, Troost FJ, et al. (2010) High temporal and inter-individual variation detected in the human ileal microbiota. Environ Microbiol 12: 3213–3227.

226. Verberkmoes NC, Russell AL, Shah M, Godzik A, Rosenquist M, et al. (2009) Shotgun metaproteomics of the human distal gut microbiota. ISME J 3: 179–189.

227. Erickson AR, Cantarel BL, Lamendella R, Darzi Y, Mongodin EF, et al. (2012) Integrated metagenomics/metaproteomics reveals human host-microbiota signatures of Crohn's disease. PLoS One 7: e49138.

228. Wang Z, Klipfell E, Bennett BJ, Koeth R, Levison BS, et al. (2011) Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. Nature 472: 57–63.

229. Pérez-Cobas AE, Gosalbes MJ, Friedrichs A, Knecht H, Artacho A, et al. (2013) Gut microbiota disturbance during antibiotic therapy: a multi-omic approach. Gut 62: 1591–1601.

230. Fritz J V, Desai MS, Shah P, Schneider JG, Wilmes P (2013) From meta-omics to causality: experimental models for human microbiome research. Microbiome 1: 14.

231. Venema K, van den Abbeele P (2013) Experimental models of the gut microbiome. Best Pract Res Clin Gastroenterol 27: 115–126.

232. Payne AN, Zihler A, Chassard C, Lacroix C (2012) Advances and perspectives in in vitro human gut fermentation modeling. Trends Biotechnol 30: 17–25.

233. Gibson GR, Cummings JH, Macfarlane GT (1988) Use of a three-stage continuous culture system to study the effect of mucin on dissimilatory sulfate reduction and methanogenesis by mixed populations of human gut bacteria. Appl Environ Microbiol 54: 2750–2755.

234. Macfarlane GT, Macfarlane S, Gibson GR (1998) Validation of a three-stage compound continuous culture system for investigating the effect of retention time on the ecology and metabolism of bacteria in the human colon. Microb Ecol 35: 180–187.

235. Cinquin C, Le Blay G, Fliss I, Lacroix C (2004) Immobilization of infant fecal microbiota and utilization in an in vitro colonic fermentation model. Microb Ecol 48: 128–138.

236. Molly K, Vande Woestyne M, Verstraete W (1993) Development of a 5-step multi-chamber reactor as a simulation of the human intestinal microbial ecosystem. Appl Microbiol Biotechnol 39: 254–258.

237. Marzorati M, van den Abbeele P, Grootaert C, de Weirdt R, Carcavilla AM, et al. (2014) Models of the human microbiota and microbiome in vitro. Adv Mol Cell Microbiol 25: 107–123.

238. Possemiers S, Bolca S, Grootaert C, Heyerick A, Decroos K, et al. (2006) The prenylflavonoid Isoxanthohumol from hops (Humulus lupulus L .) is activated into the potent phytoestrogen 8-prenylnaringenin in vitro and in the human intestine. J Nutr 136: 1862–1867.

239. Terpend K, Possemiers S, Daguet D, Marzorati M (2013) Arabinogalactan and fructo-oligosaccharides have a different fermentation profile in the Simulator of the Human Intestinal Microbial Ecosystem (SHIME ®). Environ Microbiol Rep 5: 595–603.

240. Van de Wiele T, Boon N, Possemiers S, Jacobs H, Verstraete W (2004) Prebiotic effects of chicory inulin in the simulator of the human intestinal microbial ecosystem. FEMS Microbiol Ecol 51: 143–153.

241. Molly K, Vande Woestyne M, Desmet J, Verstraete W (1994) Validation of the Simulator of the Human Intestinal Microbial Ecosystem (SHIME) Reactor Using Microorganism-associated activities. Microb Ecol Health Dis 7: 191–200.

242. Vermeiren J, van den Abbeele P, Laukens D, Vigsnaes LK, De Vos M, et al. (2012) Decreased colonization of fecal Clostridium coccoides/Eubacterium rectale species from ulcerative colitis patients in an in vitro dynamic gut model with mucin environment. FEMS Microbiol Ecol 79: 685–696.

243. Van den Abbeele P, Belzer C, Goossens M, Kleerebezem M, De Vos WM, et al. (2013) Butyrate-producing Clostridium cluster XIVa species specifically colonize mucins in an in vitro gut model. ISME J 7: 949–961.

244. Van den Abbeele P, Van de Wiele T, Verstraete W, Possemiers S (2011) The host selects mucosal and luminal associations of coevolved gut microorganisms: a novel concept. FEMS Microbiol Rev 35: 681–704.

245. Minekus M, Smeets-Peeters M, Bernalier a., Marol-Bonnin S, Havenaar R, et al. (1999) A computer-controlled system to simulate conditions of the large intestine with peristaltic mixing, water absorption and absorption of fermentation products. Appl Microbiol Biotechnol 53: 108–114.

246. Minekus M, Marteaul P, Havenaarl R, Huis JHJ (1995) A multi-compartmental dynamic computercontrolled model simulating the stomach and small intestine. Altern to Lab annimals 23: 197–209.

247. Munoz-Garcia A, Williams J (2005) Basal metabolic rate in carnivores is associated with diet after controlling for phylogeny. Physiol Biochem Zool 78: 1039–1056.

248. Hamper B, Bartges J, Kirk C, Witzel AL (2012) The unique nutritional requirements of the cat : a strict carnivore. The Cat. Elsevier Inc. pp. 236–242.

249. Zoran DL (2002) The carnivore connection to nutrition in cats. J Am Vet Med Assoc 221: 1559–1567.

250. Muegge BD, Kuczynski J, Knights D, Clemente JC, González A, et al. (2011) Diet drives convergence in gut microbiome functions across mammalian phylogeny and within humans. Science 332: 970–974.

251. Sullam KE, Essinger SD, Lozupone CA, O'Connor MP, Rosen GL, et al. (2012) Environmental and ecological factors that shape the gut bacterial communities of fish: a meta-analysis. Mol Ecol 21: 3363–3378.

252. Li Y, Guo W, Han S, Kong F, Wang C, et al. (2015) The evolution of the gut microbiota in the giant and the red pandas. Sci Rep 5: 10185.

253. Delsuc F, Metcalf JL, Wegener Parfrey L, Song SJ, González A, et al. (2014) Convergence of gut microbiomes in myrmecophagous mammals. Mol Ecol 23: 1301–1317.

254. Zhu L, Wu Q, Dai J, Zhang S, Wei F (2011) Evidence of cellulose metabolism by the giant panda gut microbiome. Proc Natl Acad Sci U S A 108: 17714–17719.

255. Nelson TM, Rogers TL, Brown M V (2013) The Gut Bacterial Community of Mammals from Marine and Terrestrial Habitats. PLoS One 8: 1–8.

256. Nelson T (2012) Factors Influencing the Gut Microbiota of Antarctic Seals.

257. Glad T, Kristiansen VF, Nielsen KM, Brusetti L, Wright A-DG, et al. (2010) Ecological characterisation of the colonic microbiota in arctic and sub-arctic seals. Microb Ecol 60: 320–330.

258. Middelbos IS, Vester Boler BM, Qu A, White BA, Swanson KS, et al. (2010) Phylogenetic characterization of fecal microbial communities of dogs fed diets with or without supplemental dietary fiber using 454 pyrosequencing. PLoS One 5: e9768.

259. Zhang H, Chen L (2010) Phylogenetic analysis of 16S rRNA gene sequences reveals distal gut bacterial diversity in wild wolves (Canis lupus). Mol Biol Rep 37: 4013–4022.

260. Suchodolski JS, Camacho J, Steiner JM (2008) Analysis of bacterial diversity in the canine duodenum, jejunum, ileum, and colon by comparative 16S rRNA gene analysis. FEMS Microbiol Ecol 66: 567–578.

261. Deng P, Swanson KS (2015) Gut microbiota of humans, dogs and cats: current knowledge and future opportunities and challenges. Br J Nutr 113 Suppl: S6–17.

262. Lavery TJ, Roudnew B, Seymour J, Mitchell JG, Jeffries T (2012) High nutrient transport and cycling potential revealed in the microbial metagenome of Australian sea lion (Neophoca cinerea) faeces. PLoS One 7: e36478.

263. Alcaide M, Messina E, Richter M, Bargiela R, Peplies J, et al. (2012) Gene sets for utilization of primary and secondary nutrition supplies in the distal gut of endangered Iberian lynx. PLoS One 7: e51521.

264. Menke S, Meier M, Melzheimer J, Kazgeba J, Mfune E (2014) Oligotyping reveals differences between gut microbiomes of free- ranging sympatric Namibian carnivores (Acinonyx jubatus ,Canis mesomelas) on a bacterial species-like level. Front Microbiol 5: 526.

265. Schwab C, Cristescu B, Northrup JM, Stenhouse GB, Gänzle M (2011) Diet and environment shape fecal bacterial microbiota composition and enteric pathogen load of grizzly bears. PLoS One 6: e27905.

266. Zhang H, Liu G, Chen L, Sha W (2015) Composition and diversity of the bacterial community in snow leopard (Uncia uncia) distal gut. Ann Microbiol 65: 703–711.

267. Glad T, Bernhardsen P, Nielsen KM, Brusetti L, Andersen M, et al. (2010) Bacterial diversity in faeces from polar bear (Ursus maritimus) in Arctic Svalbard. BMC Microbiol 10: 10.

268. Schwab C, Cristescu B, Boyce MS, Stenhouse GB, Ganzle M (2009) Bacterial populations and metabolites in the feces of free roaming and captive grizzly bears. Can J Microbiol 55: 1335–1346.

269. Wei G, Lu H, Zhou Z, Xie H, Wang A, et al. (2007) The microbial community in the feces of the giant panda (Ailuropoda melanoleuca) as determined by PCR-TGGE profiling and clone library analysis. Microb Ecol 54: 194–202.

270. Garcia-Mazcorro J, Minamoto Y (2013) Gastrointestinal microorganisms in cats and dogs: a brief review. Arch Med Vet 45: 111–124.

271. Kerr KR, Beloshapka AN, Swanson KS (2013) 2011 and 2012 Early Careers Achievement Awards : Use of genomic biology to study companion animal intestinal microbiota. J Anim Sci 91: 2504–2511.

272. Minamoto Y, Hooda S, Swanson KS, Suchodolski JS (2012) Feline gastrointestinal microbiota. Anim Heal Res Rev 13: 64–77.

273. Suchodolski JS (2011) Companion animals symposium: microbes and gastrointestinal health of dogs and cats. J Anim Sci 89: 1520–1530.

274. Kil DY, Swanson KS (2011) Companion animals symposium: role of microbes in canine and feline health. J Anim Sci 89: 1498–1505.

275. Johnston KL, Swift NC, Med BV, Hijfte MF, Rutgers HC, et al. (2001) Comparison of the bacterial flora of the duodenum in healthy cats and cats with signs of gastrointestinal tract disease. J Am Vet Med Assoc 218: 48–51.

276. Papasouliotis K, Sparkes A, Werrett G, Egan K, Gruffydd-Jones E, et al. (1998) Assessment of the bacterial flora of the proximal part of the small intestine in healthy cats, and the effect of sample collection method. Am J Vet Res 59: 48–51.

277. Terada A, Hara H, Kato S, Kimura T, Fujimori I, et al. (1993) Effect of lactosucrose (4G-beta-D-galactosylsucrose) on fecal flora and fecal putrefactive products of cats. J Vet Med Sci 55: 291–295.

278. Itoh K, Mitsuoka T, Maejima K, Hiraga C, Nakano K (1984) Comparison of fecal flora of cats based on different housing conditions with special reference to Bifidobacterium. Lab Anim 18: 280–284.

279. Osbaldiston G, Stowe E (1971) Microflora of alimentary tract of cats. Am J Vet Res 32: 1399–1405.

280. Abecia L, Hoyles L, Khoo C, Frantz N, McCartney A (2010) Effects of a novel galactooligosaccharide on the faecal microbiota of healthy and inflammatory bowel disease cats during a randomized, double-blind, cross-over feeding study. Int J Probiotics Prebiotics 5: 61–68.

281. Inness VL, McCartney a L, Khoo C, Gross KL, Gibson GR (2007) Molecular characterisation of the gut microflora of healthy and inflammatory bowel disease cats using fluorescence in situ hybridisation with special reference to Desulfovibrio spp. J Anim Physiol Anim Nutr (Berl) 91: 48–53.

282. Ritchie LE, Steiner JM, Suchodolski JS (2008) Assessment of microbial diversity along the feline intestinal tract using 16S rRNA gene analysis. FEMS Microbiol Ecol 66: 590–598.

283. Desai AR, Musil KM, Carr AP, Hill JE (2009) Characterization and quantification of feline fecal microbiota using cpn60 sequence-based methods and investigation of animal-to-animal variation in microbial population structure. Vet Microbiol 137: 120–128.

284. Garcia-Mazcorro JF, Lanerie DJ, Dowd SE, Paddock CG, Grutzner N, et al. (2011) Effect of a multi-species synbiotic formulation on fecal bacterial microbiota of healthy cats and dogs as evaluated by pyrosequencing. FEMS Microbiol Ecol 78: 542–554.

285. Handl S, Dowd SE, Garcia-Mazcorro JF, Steiner JM, Suchodolski JS (2011) Massive parallel 16S rRNA gene pyrosequencing reveals highly diverse fecal bacterial and fungal communities in healthy dogs and cats. FEMS Microbiol Ecol 76: 301–310.

286. Barry KA, Middelbos IS, Vester Boler BM, Dowd SE, Suchodolski JS, et al. (2012) Effects of dietary fiber on the feline gastrointestinal metagenome. J Proteome Res 11: 5924–5933.

287. Tun HM, Brar MS, Khin N, Jun L, Hui RK-H, et al. (2012) Gene-centric metagenomics analysis of feline intestinal microbiome using 454 junior pyrosequencing. J Microbiol Methods 88: 369–376.

288. Swanson KS, Dowd SE, Suchodolski JS, Middelbos IS, Vester BM, et al. (2011) Phylogenetic and gene-centric metagenomics of the canine intestinal microbiome reveals similarities with humans and mice. ISME J 5: 639–649.

289. Howard J, Munson L, McAloose D, Kriete M, Bush M, et al. (1993) Comparative evaluation of seminal, vaginal, and rectal bacterial flora in the cheetah and domestic cat. Zoo Biol 12: 81–96.

290. Queen E V, Marks SL, Farver TB (2012) Prevalence of selected bacterial and parasitic agents in feces from diarrheic and healthy control cats from Northern California. J Vet Intern Med 26: 54–60.

291. Suchodolski JS, Foster ML, Sohail MU, Leutenegger C, Queen E V, et al. (2015) The fecal microbiome in cats with diarrhea. PLoS One 10: e0127378.

292. Minamoto Y, Otoni CC, Steelman SM, Büyükleblebici O, Steiner JM, et al. (2015) Alteration of the fecal microbiota and serum metabolite profiles in dogs with idiopathic inflammatory bowel disease. Gut Microbes 6: 33–47.

293. Suchodolski JS, Markel ME, Garcia-Mazcorro JF, Unterer S, Heilmann RM, et al. (2012) The fecal microbiome in dogs with acute diarrhea and idiopathic inflammatory bowel disease. PLoS One 7: e51907.

294. Janeczko S, Atwater D, Bogel E, Greiter-Wilke A, Gerold A, et al. (2008) The relationship of mucosal bacteria to duodenal histopathology, cytokine mRNA, and clinical disease activity in cats with inflammatory bowel disease. Vet Microbiol 128: 178–193.

295. Suchodolski JS, Dowd SE, Wilke V, Steiner JM, Jergens AE (2012) 16S rRNA gene pyrosequencing reveals bacterial dysbiosis in the duodenum of dogs with idiopathic inflammatory bowel disease. PLoS One 7: e39333.

296. Hooda S, Vester-Boler BM, Kerr KR, Dowd SE, K.S. S (2012) The gut microbiome of kittens is affected by dietary protein: carbohydrate ratio and correlated with blood metabolite and hormone concentrations. Br J Nutr 109: 1637–1646.

297. Deusch O, O'Flynn C, Colyer A, Morris P, Allaway D, et al. (2014) Deep Illumina-based shotgun sequencing reveals dietary effects on the structure and function of the fecal microbiome of growing kittens. PLoS One 9: e101021.

298. Bermingham EN, Young W, Kittelmann S, Kerr KR, Swanson KS, et al. (2013) Dietary format alters fecal bacterial populations in the domestic cat (Felis catus). Microbiologyopen: 1–9.

299. Grześkowiak Ł, Endo A, Beasley S, Salminen S (2015) Microbiota and probiotics in canine and feline welfare. Anaerobe 34: 14–23.

300. De Vrese M, Schrezenmeir J (2008) Probiotics, Prebiotics and Synbiotics. Food Biotechnology. Berlin, Heidelberg: Springer-Verlag, Vol. 111. pp. 1–66.

301. Ramadan Z, Xu H, Laflamme D, Czarnecki-Maulden G, Li QJ, et al. (2014) Fecal microbiota of cats with naturally occurring chronic diarrhea assessed ssing 16S rRNA gene 454-pyrosequencing before and after dietary treatment. J Vet Intern Med 28: 59–65.

302. Kawata K (2008) Zoo Animal Feeding: A Natural History Viewpoint. Der Zool Garten 78: 17–42.

303. Allen ME, Ullrey DE (2004) Relationships among nutrition and reproduction and relevance for wild animals. Zoo Biol 23: 475–487.

304. Gaggìa F, Mattarelli P, Biavati B (2010) Probiotics and prebiotics in animal feeding for safe food production. Int J Food Microbiol 141: S15–S28.

305. Morris JG (2002) Idiosyncratic nutrient requirements of cats appear to be diet-induced evolutionary adaptations. Nutr Res Rev 15: 153–168.

306. Vester BM, Beloshapka AN, Middelbos IS, Burke SL, Dikeman CL, et al. (2010) Evaluation of nutrient digestibility and fecal characteristics of exotic felids fed horse- or beef-based diets: use of the domestic cat as a model for exotic felids. Zoo Biol 29: 432–448.

307. Dierenfeld ES (1993) Nutrition of captive cheetahs - food composition and blood parameters. Zoo Biol 12: 143–150.

308. Zoran DL, Buffington CAT (2011) Effects of nutrition choices and lifestyle changes on the well-being of cats, a carnivore that has moved indoors. J Am Vet Med Assoc 239: 596–606.

309. Vester Boler BM, Swanson KS, Fahey GC (2009) Nutrition of the Exotic Felid. Feedstuffs: 57–59.

310. Hooper L V, Midtvedt T, Gordon JI (2002) How host-microbial interactions shape the nutrient environment of the mammalian intestine. Annu Rev Nutr 22: 283–307.

311. Ley RE, Lozupone CA, Hamady M, Knight R, Gordon JI (2008) Worlds within worlds: evolution of the vertebrate gut microbiota. Nat Rev Microbiol 6: 776–788.

312. Barry KA, Wojcicki BJ, Middelbos IS, Vester BM, Swanson KS, et al. (2010) Dietary cellulose, fructooligosaccharides, and pectin modify fecal protein catabolites and microbial populations in adult cats. J Anim Sci 88: 2978–2987.

313. Vester BM, Dalsing BL, Middelbos IS, Apanavicius CJ, Lubbs DC, et al. (2009) Faecal microbial populations of growing kittens fed high- or moderate-protein diets. Arch Anim Nutr 63: 254–265.

314. Lubbs DC, Vester BM, Fastinger ND, Swanson KS (2009) Dietary protein concentration affects intestinal microbiota of adult cats: a study using DGGE and qPCR to evaluate differences in microbial populations in the feline gastrointestinal tract. J Anim Physiol Anim Nutr (Berl) 93: 113–121.

315. Pitcher DG, Saunders NA, Owen RJ (1989) Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett Appl Microbiol 8: 151–156.

316. Brinkman BM, Hildebrand F, Kubica M, Goosens D, Del Favero J, et al. (2011) Caspase deficiency alters the murine gut microbiome. Cell Death Dis 2: e220.

317. Fierer N, Jackson JA, Vilgalys R, Jackson RB (2005) Assessment of soil microbial community structure by use of taxon-specific quantitative PCR assays. Appl Environ Microbiol 71: 4117–4120.

318. Matsuki T, Watanabe K, Fujimoto J, Kado Y, Takada T, et al. (2004) Quantitative PCR with 16S rRNA-Gene-Targeted Species-Specific Primers for Analysis of Human Intestinal Bifidobacteria. Appl Environ Microbiol 70: 167–173.

319. Edwards U, Rogall T, Blöcker H, Emde M, Böttger EC (1989) Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene conding for 16S ribosomal RNA. Nucleic Acids Res 17: 7843–7853.

320. Coenye T, Falsen E, Vancanneyt M, Hoste B, Govan JR, et al. (1999) Classification of Alcaligenes faecalis-like isolates from the environment and human clinical samples as Ralstonia gilardii sp. nov. Int J Syst Bacteriol 49 Pt 2: 405–413.

321. Huber T, Faulkner G, Hugenholtz P (2004) Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. Bioinformatics 20: 2317–2319.

322. Gontcharova V, Youn E, Wolcott RD, Hollister EB, Gentry TJ, et al. (2010) Black box chimera check (B2C2): a windows-based software for batch depletion of chimeras from bacterial 16S datasets. Open Microbiol J 4: 47–52.

323. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, et al. (2011) MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Mol Biol Evol 28: 2731–2739.

324. Dorman N (2012) Citations. Biotechniques 52: 403–410.

325. Cole JR, Wang Q, Cardenas E, Fish J, Chai B, et al. (2009) The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res 37: D141–145.

326. Good IJ (1953) The population frequencies of species and the estimation of population parameters. Biometrika 40: 237–264.

327. Collins MD, Lawson PA, Willems A, Cordoba JJ, Fernandezgarayzabal J, et al. (1994) The phylogeny of the genus Clostridium - proposal of 5 new genera and 11 new species combinations. Int J Syst Bacteriol 44: 812–826.

328. Thomas F, Hehemann J-H, Rebuffet E, Czjzek M, Michel G (2011) Environmental and gut bacteroidetes: the food connection. Front Microbiol 2: 93.

329. Tremaroli V, Bäckhed F (2012) Functional interactions between the gut microbiota and host metabolism. Nature 489: 242–249.

330. Swanson KS, Dowd SE, Suchodolski JS, Middelbos IS, Vester BM, et al. (2011) Phylogenetic and gene-centric metagenomics of the canine intestinal microbiome reveals similarities with humans and mice. ISME J 5: 639–649.

331. Ritchie LE, Burke KF, Garcia-Mazcorro JF, Steiner JM, Suchodolski JS (2010) Characterization of fecal microbiota in cats using universal 16S rRNA gene and group-specific primers for Lactobacillus and Bifidobacterium spp. Vet Microbiol 144: 140–146.

332. Schwab C, Gänzle M (2011) Comparative analysis of fecal microbiota and intestinal microbial metabolic activity in captive polar bears. Can J Microbiol 57: 177–185.

333. Hayashi H, Sakamoto M, Kitahara M, Benno Y (2006) Diversity of the Clostridium coccoides group in human fecal microbiota as determined by 16S rRNA gene library. FEMS Microbiol Lett 257: 202–207.

334. Hoskins LC (1992) Mucin degradation in the human gastrointestinal tract and its significance to enteric microbial ecology. Eur J Gastroenterol Hepatol 5: 205–213.

335. Liu C, Finegold SM, Song Y, Lawson PA (2008) Reclassification of Clostridium coccoides, Ruminococcus hansenii, Ruminococcus hydrogenotrophicus, Ruminococcus luti, Ruminococcus productus and Ruminococcus schinkii as Blautia coccoides gen. nov., comb. nov., Blautia hansenii comb. nov., Blautia hydroge. Int J Syst Evol Microbiol 58: 1896–1902.

336. Barcenilla A, Pryde SE, Martin JC, Duncan H, Stewart CS, et al. (2000) Phylogenetic relationships of butyrate-producing bacteria from the human gut. Appl Environ Microbiol 66: 1654–1661.

337. Meijer K, de Vos P, Priebe MG (2010) Butyrate and other short-chain fatty acids as modulators of immunity: what relevance for health? Curr Opin Clin Nutr Metab Care 13: 715–721.

338. Kitahara M, Takamine F, Imamura T, Benno Y (2001) Clostridium hiranonis sp. nov., a human intestinal bacterium with bile acid 7alpha-dehydroxylating activity. Int J Syst Evol Microbiol 51: 39–44.

339. Zentek J, Fricke S, Hewicker-trautwein M, Ehinger B, Amtsberg G, et al. (2004) Dietary protein source and manufacturing processes affect macronutrient digestibility, fecal consistency, and presence of fecal Clostridium perfringens in adult dogs. J Nutr 134: 2158S–2161S.

340. Kolida S, Tuohy K, Gibson GR (2007) Prebiotic effects of inulin and oligofructose. Br J Nutr 87: S193–S197.

341. Dethlefsen L, Huse S, Sogin ML, Relman DA (2008) The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. PLoS Biol 6: e280.

342. Krogius-Kurikka L, Kassinen A, Paulin L, Corander J, Mäkivuokko H, et al. (2009) Sequence analysis of percent G+C fraction libraries of human faecal bacterial DNA reveals a high number of Actinobacteria. BMC Microbiol 9: 68.

343. Zentek J, Marquart B, Pietrzak T, Ballèvre O, Rochat F (2003) Dietary effects on bifidobacteria and Clostridium perfringens in the canine intestinal tract. J Anim Physiol Anim Nutr (Berl) 87: 397–407.

344. Endo A, Futagawa-Endo Y, Dicks LMT (2010) Diversity of Lactobacillus and Bifidobacterium in feces of herbivores, omnivores and carnivores. Anaerobe 16: 590–596.

345. King J (1998) Shigella flexneri: A practical review for zoo personnel. Zoo Biol 17: 59–76.

346. Green CE (2012) Infectious diseases of the dog and cat. 4th ed. Stringer S, editor Philadephia: Saunders.

347. Whitehouse-Tedd K, Lefebre S, Janssens GPJ (2012) A global survey of captive cheetah diet, faecal consistency and gastrointestinal disease. Proceedings of the 14th Annual BIAZA Research Symposium. p. 9.

348. Kohl KD, Amaya J, Passement CA, Dearing MD, McCue MD (2014) Unique and shared responses of the gut microbiota to prolonged fasting: a comparative study across five classes of vertebrate hosts. FEMS Microbiol Ecol 90: 883–894.

349. Amato KR, Yeoman CJ, Kent A, Righini N, Carbonero F, et al. (2013) Habitat degradation impacts black howler monkey (Alouatta pigra) gastrointestinal microbiomes. ISME J 7: 1344–1353.

350. Hayward MW, Hofmeyr M, O'Brien J, Kerley GIH (2006) Prey preferences of the cheetah (Acinonyx jubatus) (Felidae: Carnivora): morphological limitations or the need to capture rapidly consumable prey before kleptoparasites arrive? J Zool 270: 615–627.

351. Becker AAMJ, Hesta M, Hollants J, Janssens GPJ, Huys G (2014) Phylogenetic analysis of faecal microbiota from captive cheetahs reveals underrepresentation of Bacteroidetes and Bifidobacteriaceae. BMC Microbiol 14: 43.

352. Costello EK, Lauber CL, Hamady M, Fierer N, Jeffrey I, et al. (2009) Bacterial community variation in human body habitats across space and time. Science (80-) 326: 1694–1697.

353. Vester BM, Middelbos IS, Burke SL, Dikeman CL, Simmons LG, et al. (2008) Fecal microbial populations of large captive exotic felids and domestic cats fed beef-and horse-based raw diets. Proceedings of the Comparative Nutrition Society. pp. 243–244.

354. Temmerman R, Scheirlinck I, Huys G, Swings J (2003) Culture-Independent Analysis of Probiotic Products by Denaturing Gradient Gel Electrophoresis. Appl Environ Microbiol 69: 220–226.

355. Kim O-S, Cho Y-J, Lee K, Yoon S-H, Kim M, et al. (2012) Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. Int J Syst Evol Microbiol 62: 716–721.

356. Wittebolle L, Vervaeren H, Verstraete W, Boon N (2008) Quantifying community dynamics of nitrifiers in functionally stable reactors. Appl Environ Microbiol 74: 286–293.

357. Benjamini Y, Hochberg Y (2000) On the Adaptive Control of the False Discovery Rate in Multiple Testing With Independent Statistics. J Educ Behav Stat 25: 60–83.

358. Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative CT method. Nat Protoc 3: 1101–1108.

359. Pfaffl MW (2004) Quantification strategies in real-time PCR. In: Bustin SA, editor. A-Z of quantitative PCR. CA, USA: La Jolla. pp. 87–112.

360. Marker LL (2014) International Cheetah Studbook 2013. Marker LL, editor Cheetah Conservation Fund.

361. Degnan PH, Pusey AE, Lonsdorf E V, Goodall J, Wroblewski EE, et al. (2012) Factors associated with the diversification of the gut microbial communities within chimpanzees from Gombe National Park. PNAS 109: 13034–13039.

362. Schloss PD, Schubert AM, Zackular JP, Iverson KD, Young VB, et al. (2012) Stabilization of the murine gut microbiome following weaning. Gut Microbes 3: 383–393.

363. Buddington RK, Sangild PT (2011) Companion animals symposium: development of the mammalian gastrointestinal tract, the resident microbiota, and the role of diet in early life. J Anim Sci 89: 1506–1519.

364. Biagi E, Candela M, Fairweather-Tait S, Franceschi C, Brigidi P (2012) Aging of the human metaorganism: the microbial counterpart. Age (Omaha) 34: 247–267.

365. Claesson MJ, Cusack S, O'Sullivan O, Greene-Diniz R, de Weerd H, et al. (2011) Composition, variability, and temporal stability of the intestinal microbiota of the elderly. PNAS 108: 4586–4591.

366. Biagi E, Nylund L, Candela M, Ostan R, Bucci L, et al. (2010) Through ageing, and beyond: gut microbiota and inflammatory status in seniors and centenarians. PLoS One 5: e10667.

367. Fahey GC, Barry KA, Swanson KS (2008) Age-related changes in nutrient utilization by companion animals. Annu Rev Nutr 28: 425–445.

368. Simpson JM, Martineau B, Jones WE, Ballam JM, Mackie RI (2002) Characterization of fecal bacterial populations in canines: effects of age, breed and dietary fiber. Microb Ecol 44: 186–197.

369. Collins MD, Lawson PA, Willems A, Cordoba JJ, Fernandez-Garayzabal J, et al. (1994) The phylogeny of the genus Clostridium: proposal of five new genera and eleven new species combinations. Int J Syst Bacteriol 44: 812–826.

370. Silva ROS, D'Elia ML, Tostes Teixeira EP, Pereira PLL, de Magalhães Soares DF, et al. (2014) Clostridium difficile and Clostridium perfringens from wild carnivore species in Brazil. Anaerobe 28: 207–211.

371. Jores J, Derocher AE, Staubach C, Aschfalk A (2008) Occurrence and Prevalence of Clostridium perfringens in Polar Bears. 44: 155–158.

372. Zeira O, Briola C, Konar M, Dumas MP, Wrzosek MA, et al. (2012) Suspected neurotoxicity due to Clostridium perfringens type B in a tiger (Panthera tigris). J Zoo Wildl Med 43: 666–669.

373. Zhang Y, Hou Z, Ma J (2012) Hemorrhagic enterocolitis and death in two felines (Panthera tigris altaica and Panthera leo) associated with Clostridium perfringens type A. J Zoo Wildl Med 43: 394–396.

374. Greiner T, Bäckhed F (2011) Effects of the gut microbiota on obesity and glucose homeostasis. Trends Endocrinol Metab 22: 117–123.

375. Plantinga EA, Bosch G, Hendriks WH (2011) Estimation of the dietary nutrient profile of free-roaming feral cats: possible implications for nutrition of domestic cats. Br J Nutr 106: S35–48.

376. Dierenfeld ES, Alcorn HL, Jacobsen KL (2002) Nutrient composition of whole vertebrate prey (excluding fish) fed in zoos. U.S. Dept. of Agriculture, Agricultural Research Service, National Agricultural Library, Animal Welfare Information Center.

377. Bolnick DI, Snowberg LK, Hirsch PE, Lauber CL, Org E, et al. (2014) Individual diet has sex-dependent effects on vertebrate gut microbiota. Nat Commun 5: 4500.

378. Nelson TM, Rogers TL, Carlini AR, Brown M V (2013) Diet and phylogeny shape the gut microbiota of Antarctic seals: a comparison of wild and captive animals. Environ Microbiol 15: 1132–1145.

379. Turnbaugh PJ, Ridaura VK, Faith JJ, Rey FE, Gordon JI (2009) The effect of diet on the human gut microbiome: A metagenomic analysis in humanized gnotobiotic mice. Sci Transl Med 1: 6ra14.

380. Barc MC, Bourlioux F, Charrin-sarnel C, Janoir C, Doré J, et al. (2004) Effect of Amoxicillin-Clavulanic Acid on Human Fecal Flora in a Gnotobiotic Mouse Model Assessed with Fluorescence Hybridization Using Group-Specific 16S rRNA Probes in Combination with Flow Cytometry. Antimicrob Agents Chemother 48: 1365–1368.

381. Větrovský T, Baldrian P (2013) The variability of the 16S rRNA gene in bacterial genomes and its consequences for bacterial community analyses. PLoS One 8: e57923.

382. Smalla K, Oros-Sichler M, Milling A, Heuer H, Baumgarte S, et al. (2007) Bacterial diversity of soils assessed by DGGE, T-RFLP and SSCP fingerprints of PCR-amplified 16S rRNA gene fragments: do the different methods provide similar results? J Microbiol Methods 69: 470–479.

383. Rinttilä T, Kassinen A, Malinen E, Krogius L, Palva A (2004) Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. J Appl Microbiol 97: 1166–1177.

384. Matsuki T, Watanabe K, Fujimoto J, Takada T, Matsumoto K, et al. (2002) Development of 16S rRNA-gene-targeted group-specific primers for the detection and identification of predominant bacteria in human feces. Appl Environ Microbiol 68: 5445–5451.

385. Masco L, Vanhoutte T, Temmerman R, Swings J, Huys G (2007) Evaluation of real-time PCR targeting the 16S rRNA and recA genes for the enumeration of bifidobacteria in probiotic products. Int J Food Microbiol 113: 351–357.

386. Karlsson F, Tremaroli V, Nielsen J, Bäckhed F (2013) Assessing the human gut microbiota in metabolic diseases. Diabetes 62: 3341–3349.

387. Van Dijk EL, Auger H, Jaszczyszyn Y, Thermes C (2014) Ten years of next-generation sequencing technology. Trends Genet 30: 418–426.

388. Claesson MJ, Wang Q, O'Sullivan O, Greene-Diniz R, Cole JR, et al. (2010) Comparison of two next-generation sequencing technologies for resolving highly complex microbiota composition using tandem variable 16S rRNA gene regions. Nucleic Acids Res 38: e200.

389. Lazarevic V, Whiteson K, Huse S, Hernandez D, Farinelli L, et al. (2009) Metagenomic study of the oral microbiota by Illumina high-throughput sequencing. J Microbiol Methods 79: 266–271.

390. Kuczynski J, Lauber CL, Walters W a, Parfrey LW, Clemente JC, et al. (2012) Experimental and analytical tools for studying the human microbiome. Nat Rev Genet 13: 47–58.

391. Metzker ML (2010) Sequencing technologies - the next generation. Nat Rev Genet 11: 31–46.

392. Mardis ER (2008) The impact of next-generation sequencing technology on genetics. Trends Genet 24: 133–141.

393. Morozova O, Marra M a (2008) Applications of next-generation sequencing technologies in functional genomics. Genomics 92: 255–264.

394. Pop M, Salzberg SL (2008) Bioinformatics challenges of new sequencing technology. Trends Genet 24: 142–149.

395. Ding C, He J (2012) Molecular techniques in the biotechnological fight against halogenated compounds in anoxic environments. Microb Biotechnol 5: 347–367.

396. Kautz S, Rubin BER, Russell JA, Moreau CS (2013) Surveying the microbiome of ants: comparing 454 pyrosequencing with traditional methods to uncover bacterial diversity. Appl Environ Microbiol 79: 525–534.

397. Li P, Jiang D, Li B, Dai X, Wang Y, et al. (2014) Comparative survey of bacterial and archaeal communities in high arsenic shallow aquifers using 454 pyrosequencing and traditional methods. Ecotoxicology 23: 1878–1889.

398. Delgado S, Rachid CTCC, Fernández E, Rychlik T, Alegría A, et al. (2013) Diversity of thermophilic bacteria in raw, pasteurized and selectively-cultured milk, as assessed by culturing, PCR-DGGE and pyrosequencing. Food Microbiol 36: 103–111.

399. Leite a MO, Mayo B, Rachid CTCC, Peixoto RS, Silva JT, et al. (2012) Assessment of the microbial diversity of Brazilian kefir grains by PCR-DGGE and pyrosequencing analysis. Food Microbiol 31: 215–221.

400. Ling Z, Kong J, Jia P, Wei C, Wang Y, et al. (2010) Analysis of oral microbiota in children with dental caries by PCR-DGGE and barcoded pyrosequencing. Microb Ecol 60: 677–690.

401. Becker AAMJ, Janssens GPJ, Snauwaert C, Hesta M, Huys G (2015) Integrated Community Profiling Indicates Long-Term Temporal Stability of the Predominant Faecal Microbiota in Captive Cheetahs. PLoS One 10: e0123933.

402. Vanhoutte T, Huys G, Brandt E, Swings J (2004) Temporal stability analysis of the microbiota in human feces by denaturing gradient gel electrophoresis using universal and group-specific 16S rRNA gene primers. FEMS Microbiol Ecol 48: 437–446.

403. Bohorquez L, Delgado-Serrano L, Lopez G, Osorio-Forero C, Klepac-Ceraj V, et al. (2012) In-depth characterization via complementing culture-independent approaches of the microbial community in an acidic hot spring of the Colombian Andes. Environ Microbiol 63: 103–115.

404. Camarinha-Silva A, Jáuregui R, Chaves-Moreno D, Oxley AP a, Schaumburg F, et al. (2014) Comparing the anterior nare bacterial community of two discrete human populations using Illumina amplicon sequencing. Environ Microbiol 16: 2939–2952.

405. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, et al. (2009) Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 75: 7537–7541.

406. Wang Q, Garrity GM, Tiedje JM, Cole JR (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 73: 5261–5267.

407. Wittebolle L, Marzorati M, Clement L, Balloi A, Daffonchio D, et al. (2009) Initial community evenness favours functionality under selective stress. Nature 458: 623–626.

408. Marzorati M, Wittebolle L, Boon N, Daffonchio D, Verstraete W (2008) How to get more out of molecular fingerprints: practical tools for microbial ecology. Environ Microbiol 10: 1571–1581.

409. Lozupone C, Lladser ME, Knights D, Stombaugh J, Knight R (2011) UniFrac: an effective distance metric for microbial community comparison. ISME J 5: 169–172.

410. Logares R, Audic S, Bass D, Bittner L, Boutte C, et al. (2014) Patterns of rare and abundant marine microbial eukaryotes. Curr Biol 24: 813–821.

411. Pedrós-Alió C (2012) The Rare Bacterial Biosphere. Ann Rev Mar Sci 4: 449–466.

412. R Core Team (2013) R: A language and environment for statisticla computing. R Found Stat Comput Vienna, Austria.

413. Bacci G, Bani A, Bazzicalupo M, Ceccherini MT, Galardini M, et al. (2015) Evaluation of the Performances of Ribosomal Database Project (RDP) Classifier for Taxonomic Assignment of 16S rRNA Metabarcoding Sequences Generated from Illumina-Solexa NGS. J genomics 3: 36–39.

414. Bartram AK, Lynch MDJ, Stearns JC, Moreno-Hagelsieb G, Neufeld JD (2011) Generation of multimillion-sequence 16S rRNA gene libraries from complex microbial communities by assembling paired-end illumina reads. Appl Environ Microbiol 77: 3846–3852.

415. Lynch MDJ, Neufeld JD (2015) Ecology and exploration of the rare biosphere. Nat Rev Microbiol 13: 217–229.

416. Hill JE, Fernando WMU, Zello G a, Tyler RT, Dahl WJ, et al. (2010) Improvement of the representation of bifidobacteria in fecal microbiota metagenomic libraries by application of the cpn60 universal primer cocktail. Appl Environ Microbiol 76: 4550–4552.

417. Pilloni G, Granitsiotis MS, Engel M, Lueders T (2012) Testing the limits of 454 pyrotag sequencing: reproducibility, quantitative assessment and comparison to T-RFLP fingerprinting of aquifer microbes. PLoS One 7: e40467.

418. Vaz-Moreira I, Egas C, Nunes OC, Manaia CM (2013) Bacterial diversity from the source to the tap: a comparative study based on 16S rRNA gene-DGGE and culture-dependent methods. FEMS Microbiol Ecol 83: 361–374.

419. Shade A, Jones SE, Caporaso JG, Handelsman J, Knight R, et al. (2014) Conditionally rare taxa disproportionately contribute to temporal changes in microbial diversity. MBio 5: e01371–14.

420. Xu J, Gordon JI (2003) Honor thy symbionts. Proc Natl Acad Sci U S A 100: 10452–10459.

421. Moeller AH, Ochman H (2015) Factors that drive variation among gut microbial communities. Gut Microbes 4: 403–408.

422. Suchodolski JS (2011) Intestinal microbiota of dogs and cats: a bigger world than we thought. Vet Clin North Am Small Anim Pract 41: 261–272.

423. Wienemann T, Schmitt-Wagner D, Meuser K, Segelbacher G, Schink B, et al. (2011) The bacterial microbiota in the ceca of Capercaillie (Tetrao urogallus) differs between wild and captive birds. Syst Appl Microbiol 34: 542–551.

424. Nakamura N, Amato KR, Garber P, Estrada A, Mackie RI, et al. (2011) Analysis of the hydrogenotrophic microbiota of wild and captive black howler monkeys (Alouatta pigra) in palenque national park, Mexico. Am J Primatol 73: 909–919.

425. Xenoulis PG, Gray PL, Brightsmith D, Palculict B, Hoppes S, et al. (2010) Molecular characterization of the cloacal microbiota of wild and captive parrots. Vet Microbiol 146: 320–325.

426. Scupham AJ, Patton TG, Bent E, Bayles DO (2008) Comparison of the cecal microbiota of domestic and wild turkeys. Microb Ecol 56: 322–331.

427. Uenishi G, Fujita S, Ohashi G, Kato A, Shino Y, et al. (2007) Molecular Analyses of the Intestinal Microbiota of Chimpanzees in the Wild and in Captivity. Amrican J Primatol 69: 367–376.

428. Becker A, Van de Wiele T, Jauregui R, Vilchez-Vargas R, Pieper D, et al. (n.d.) The faecal microbiota of captive cheetahs: concordance of global phylogenetic diversity and temporal stability patterns derived from conventional microbial ecology tools versus next-generation sequencing. (submitted to PLoS One).

429. Ochman H, Worobey M, Kuo C-H, Ndjango J-BN, Peeters M, et al. (2010) Evolutionary relationships of wild hominids recapitulated by gut microbial communities. PLoS Biol 8: e1000546.

430. Rangel-Negrín a., Alfaro JL, Valdez R a., Romano MC, Serio-Silva JC (2009) Stress in Yucatan spider monkeys: effects of environmental conditions on fecal cortisol levels in wild and captive populations. Anim Conserv 12: 496–502.

431. Kohl KD, Skopec MM, Dearing MD (2014) Captivity results in disparate loss of gut microbial diversity in closely related hosts. Conserv Physiol 2: 1–11.

432. Amato KR (2013) Co-evolution in context: The importance of studying gut microbiomes in wild animals. Microbiome Sci Med 1: 10–29.

433. Hooper L V, Midtvedt T, Gordon JI (2002) How host-microbial interactions shape the nutrient environment of the mammalian intestine. Annu Rev Nutr 22: 283–307.

434. Kohl KD, Dearing MD (2014) Wild-caught rodents retain a majority of their natural gut microbiota upon entrance into captivity. Environ Microbiol Rep 6: 191–195.

435. De la Fe C, Rodríguez JM, Ramírez G a, Hervás J, Gil J, et al. (2006) Sudden death associated with Clostridium sordellii in captive lions (Panthera leo). Vet Pathol 43: 370–374.

436. Schneeberg A, Rupnik M, Neubauer H, Seyboldt C (2012) Prevalence and distribution of Clostridium difficile PCR ribotypes in cats and dogs from animal shelters in Thuringia, Germany. Anaerobe 18: 484–488.

437. Kerr KR, Dowd SE, Swanson KS (2014) Faecal microbiota of domestic cats fed raw whole chicks v. an extruded chicken-based diet. J Nutr Sci 3: e22.

438. Eren AM, Sogin ML, Morrison HG, Vineis JH, Fisher JC, et al. (2015) A single genus in the gut microbiome reflects host preference and specificity. ISME J 9: 90–100.

439. Lopetuso LR, Scaldaferri F, Petito V, Gasbarrini A (2013) Commensal Clostridia: leading players in the maintenance of gut homeostasis. Gut Pathog 5: 23.

440. Van den Abbeele P, Roos S, Eeckhaut V, MacKenzie DA, Derde M, et al. (2012) Incorporating a mucosal environment in a dynamic gut model results in a more representative colonization by lactobacilli. Microb Biotechnol 5: 106–115.

441. Tanner S a, Zihler Berner A, Rigozzi E, Grattepanche F, Chassard C, et al. (2014) In vitro continuous fermentation model (PolyFermS) of the swine proximal colon for simultaneous testing on the same gut microbiota. PLoS One 9: e94123.

442. MacDonald M, Rogers Q, Morris J (1984) Nutrition of the domestic cat, a mammalian carnivore. Annu Rev Nutr 4: 521–562.

443. Vester Boler BM, Swanson KS, Fahey GC (2009) Nutrition of the Exotic Felid. Feedstuffs: 57–59.

444. Van de Wiele T, van den Abbeele P, Ossieur W, Possemiers S, Marzorati M (2015) Chapter 27: The Simulator of the Human Intestinal Microbial Ecosystem (SHIME). In: Verhoeckx K, Cotter P, Lopez-Exposito I, Kleiveland C, Lea T, et al., editors. The Impact of Food Bioactives on Health: In vitro and ex vivo models. Springer London. pp. 305–318.

445. Vanden Bussche J, Marzorati M, Laukens D, Vanhaecke L (2015) A validated high resolution mass spectrometry-based approach for metabolomic fingerprinting of the human gut phenotype. Anal Chem accepted.

446. Possemiers S, Verthé K, Uyttendaele S, Verstraete W (2004) PCR-DGGE-based quantification of stability of the microbial community in a simulator of the human intestinal microbial ecosystem. FEMS Microbiol Ecol 49: 495–507.

447. Chandler ML, Guilford G, Lawoko CR (1997) Radiopaque markers to evaluate gastric emptying and small intestinal transit time in healthy cats. J Vet Intern Med 11: 361–364.

448. National Research Council (2006) Nutrient Requirements of Dogs and Cats. The National Academies Press, editor Washington, DC.

449. Brosey BP, Hill RC, Scott KC (2000) Gastrointestinal volatile fatty acid concentrations and pH in cats. Am J Vet Res 61: 359–361.

450. De Weirdt R, Possemiers S, Vermeulen G, Moerdijk-Poortvliet TCW, Boschker HTS, et al. (2010) Human faecal microbiota display variable patterns of glycerol metabolism. FEMS Microbiol Ecol 74: 601–611.

451. De Boever P, Deplancke B, Verstraete W (2000) Fermentation by gut microbiota cultured in a simulator of the human intestinal microbial ecosystem is improved by supplementing a soygerm powder. J Nutr 130: 2599–2606.

452. Kosmides AK, Kamisoglu K, Calvano SE, Corbett SA, Androulakis IP (2013) Metabolomic Fingerprinting: Challenges and Opportunities. Crit Rev Biomed Eng 41: 205–221.

453. Eriksson L, Trygg J, Wold S (2008) CV-ANOVA for significance testing of PLS and OPLS® models. J Chemom 22: 594–600.

454. Xia J, Sinelnikov I V., Han B, Wishart DS (2015) MetaboAnalyst 3.0--making metabolomics more meaningful. Nucleic Acids Res: 1–7.

455. Wiklund S, Johansson E, Sjo L, Shockcor JP, Gottfries J, et al. (2008) Visualization of GC / TOF-MS-Based Metabolomics Data for Identification of Biochemically Interesting Compounds Using OPLS Class Models. 80: 115–122.

456. Barupal DK, Haldiya PK, Wohlgemuth G, Kind T, Kothari SL, et al. (2012) MetaMapp: mapping and visualizing metabolomic data by integrating information from biochemical pathways and chemical and mass spectral similarity. BMC Bioinformatics 13: 99.

457. Smilde AK, Jansen JJ, Hoefsloot HCJ, Lamers R-J a N, van der Greef J, et al. (2005) ANOVA-simultaneous component analysis (ASCA): a new tool for analyzing designed metabolomics data. Bioinformatics 21: 3043–3048.

458. Xia J, Sinelnikov I V, Wishart DS (2011) MetATT: a web-based metabolomics tool for analyzing time-series and two-factor data sets. Bioinformatics: 2–3.

459. Lin Y-K, Kuan C-Y (2010) Development of 4-hydroxyproline analysis kit and its application to collagen quantification. Food Chem 119: 1271–1277.

460. Davila A-M, Blachier F, Gotteland M, Andriamihaja M, Benetti P-H, et al. (2013) Intestinal luminal nitrogen metabolism: role of the gut microbiota and consequences for the host. Pharmacol Res 68: 95–107.

461. Aguirre M, Ramiro-Garcia J, Koenen ME, Venema K (2014) To pool or not to pool? Impact of the use of individual and pooled fecal samples for in vitro fermentation studies. J Microbiol Methods 107: 1–7.

462. Shoulders MD, Raines RT (2009) Collagen Structure and Stability. Annu Rev Biochem 78: 929–958.

463. Grootaert C, van den Abbeele P, Marzorati M, Broekaert WF, Courtin CM, et al. (2009) Comparison of prebiotic effects of arabinoxylan oligosaccharides and inulin in a simulator of the human intestinal microbial ecosystem. FEMS Microbiol Ecol 69: 231–242.

464. Harkness MLR, Harkness RD, Venni MF (1978) Digestion of native collagen in the gut: 240–243.

465. Fierer N, Ferrenberg S, Flores GE, González A, Kueneman J, et al. (2012) From Animalcules to an Ecosystem: Application of Ecological Concepts to the Human Microbiome. Annu Rev Ecol Evol Syst 43: 137–155.

466. Relman D (2012) The human microbiome: ecosystem resilience and health. Nutr Rev 70 Suppl 1: S2–9.

467. Martínez I, Muller CE, Walter J (2013) Long-term temporal analysis of the human fecal microbiota revealed a stable core of dominant bacterial species. PLoS One 8: e69621.

468. Mcdonald D, Hornig M, Lozupone C, Debelius J, Gilbert JA, et al. (2015) Towards large-cohort comparative studies to define the factors influencing the gut microbial community structure of ASD patients. Microb Ecol Health Dis 26: 26555.

469. Graf D, Cagno R Di, Fa F, Flint H, Nyman M, et al. (2015) Contribution of diet to the composition of the human gut microbiota °. Microb Ecol Health Dis 1: 1–11.

470. Clauss M, Kleffner H, Kienzle E (2010) Carnivorous mammals: nutrient digestibility and energy evaluation. Zoo Biol 29: 687–704.

471. Hang I, Rinttila T, Zentek J, Kettunen A, Alaja S, et al. (2012) Effect of high contents of dietary animal-derived protein or carbohydrates on canine faecal microbiota. BMC Vet Res 8: 90.

472. Stenglein JL, DE Barba M, Ausband DE, Waits LP (2010) Impacts of sampling location within a faeces on DNA quality in two carnivore species. Mol Ecol Resour 10: 109–114.

473. Wu GD, Lewis JD, Hoffmann C, Chen Y-Y, Knight R, et al. (2010) Sampling and pyrosequencing methods for characterizing bacterial communities in the human gut using 16S sequence tags. BMC Microbiol 10: 206.

474. Gray M a, Pratte Z a, Kellogg C a (2012) Comparison of DNA preservation methods for environmental bacterial community samples. FEMS Microbiol Ecol.

475. Nechvatal JM, Ram JL, Basson MD, Namprachan P, Niec SR, et al. (2008) Fecal collection, ambient preservation, and DNA extraction for PCR amplification of bacterial and human markers from human feces. J Microbiol Methods 72: 124–132.

476. Von Bodman SB, Willey JM, Diggle SP (2008) Cell-Cell Communication in Bacteria: United We Stand. J Bacteriol 190: 4377–4391.

477. Worley B, Powers R (2013) Multivariate Analysis in Metabolomics: 92–107.

478. Mahadevan S, Shah SL, Marrie TJ, Slupsky CM (2008) Analysis of Metabolomic Data Using Support Vector Machines. Anaytical Chem 80: 7562–7570.

479. Dettmer K, Aronov PA, Hammock BD (2007) Mass spectrometry-based metabolomics. Mass Spectrom Rev 26: 51–78.

480. Saric J, Wang Y, Li J, Coen M, Utzinger J, et al. (2008) Species Variation in the Fecal Metabolome Gives Insight into Differential Gastrointestinal Function research articles: 352–360.

481. Marcobal A, Kashyap PC, Nelson TA, Aronov PA, Donia MS, et al. (2013) A metabolomic view of how the human gut microbiota impacts the host metabolome using humanized and gnotobiotic mice. ISME J 7: 1933–1943.

482. Armougom F, Raoult D (2009) Exploring Microbial Diversity using 16S rRNA high-throughput methods. J Comput Sci Syst Biol 2: 74–92.

483. Woese C (1987) Bacterial evolution. Microbiol Rev 51: 221–271.

484. Robinson CJ, Bohannan BJM, Young VB (2010) From structure to function: the ecology of host-associated microbial communities. Microbiol Mol Biol Rev 74: 453–476.

485. Case RJ, Boucher Y, Dahllöf I, Holmström C, Doolittle WF, et al. (2007) Use of 16S rRNA and rpoB genes as molecular markers for microbial ecology studies. Appl Environ Microbiol 73: 278–288.

486. Thompson CC (2004) Use of recA as an alternative phylogenetic marker in the family Vibrionaceae. Int J Syst Evol Microbiol 54: 919–924.

487. Hassan AA, Khan IU, Abdulmawjood A, Lämmler C (2003) Inter- and intraspecies variations of the 16S – 23S rDNA intergenic spacer region. Syst Appl Microbiol 26: 97–103.

Curriculum Vitae

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COMPETENCES

Initiator, communicator & inspirator
Motivated and creative team-player
Eager to learn and work in an international environment

WORK EXPERIENCE

2010-2015	**Ph.D. student in Veterinary Sciences** Laboratory of Microbiology | Faculty of Sciences, Ghent University, Belgium | Promotor: Prof. dr. Geert Huys Laboratory of Animal Nutrition | Faculty of Veterinary Medicine, Ghent University, Belgium | Promotors: Prof. dr. ir. Geert PJ Janssens & Prof. dr. Myriam Hesta, dipl ECVCN

EDUCATIONAL BACKGROUND

2004-2010	**Master in Veterinary Sciences, veterinary degree (magna cum laude)** Faculty of Veterinary Medicine, Ghent University, Belgium Dissertation: 'Salivary proline-rich proteins in free-ranging zebu cattle (*Bos indicus*) as marker of habitat degradation in Ethiopia' Promotor: Prof. dr. ir. Geert PJ Janssens & Dr. Yisehak Kechero
2006-2008	**Qualified Teachers Training for Higher Secondary Education (cum laude)** Faculty of Psychology and Pedagogic Science, Ghent University, Belgium Internship at Freinetatheneum De Wingerd, Ghent | Second and thirth grade biology lessons
1998-2004	**Secondary school degree, Latin-Mathematics** Sint-Jozefinstituut & Pleinschool, Kortrijk

ADDITIONAL EDUCATION

2010-2015	**Doctoral schools program of Life Sciences and Medicine** Ghent University, Belgium Molecular Microbial Ecology | Prof. dr. Geert Huys Basics of Statistical Inference | Prof. dr. M. Ysebaert Analysis of Variance (ANOVA) | Prof. dr. M. Ysebaert Advanced Academic English Writing skills | L. Van Vlem Advanced Academic English Conference skills | L. Van Vlem Research plan and Proposal Writing | Prof. dr. K. De Bossschere and dr. D. De Groote 'The importance of scientific communication' course 'Personal Effectiveness' course EAZA Academy Feeding Herbivores Workshop (EAZA Nutrition Conference, January 2012, Zürich)
2008	**Infocycle Belgian Development Agency** Training on international cooperation and North-South relations organised by the Belgian Development Agency, Brussels, Belgium

SCIENTIFIC OUTPUT

A1-PUBLICATIONS

- **Becker AAMJ**, Hesta M, Hollants J, Janssens GPJ, Huys G (2014). Phylogenetic analysis of faecal microbiota from captive cheetahs reveals underrepresentation of Bacteroidetes and Bifidobacteriaceae. BMC Microbiology 14:43
- **Becker AAMJ**, Janssens GPJ, Snauwaert C, Hesta M, Huys G (2015) Integrated community profiling indicates long-term temporal stability of the predominant faecal microbiota in captive cheetahs PloS One 10 e:0123933
- **Becker AAMJ**, Van de Wiele T, Jauregui R, Vilchez-Vargas R, Pieper DH, Hesta M, Janssens GPJ, Huys G. The faecal microbiota of captive cheetahs: concordance of global phylogenetic diversity and temporal stability patterns derived from conventional molecular tools versus next-generation sequencing. (*submitted*)
- **Becker AAMJ**, Hesta M, Van de Wiele T, Vanden Bussche J, Vanhaecke L, Janssens GPJ, Huys G. Adapting a dynamic simulator of the human intestinal microbial ecosystem to an *in vitro* model mimicking the microbial composition and metabolic homeostasis within the gut environment of the cheetah, a strict carnivore with vulnerable status. (*manuscript in preparation*)
- Brinkman B, **Becker A**, Ayiseh R, Hildebrand F, Raes J, Huys G, Vandenabeele P (2013) Gut microbiota affects sensitivity to acute DSS-induced colitis independently of host genotype. Inflammatory Bowel Diseases 19, 2560-2567.
- Yisehak K, **Becker A**, Rothman JM, Dierenfeld ES, Marescau B, Bosch G, Hendriks WH, Janssens GPJ (2012) Amino acid profile of salivary proteins and plasmatic trace mineral response to dietary condensed tannins in free-ranging zebu cattle (*Bos indicus*) as a marker of habitat degradation. Livestock Science 144, 275-280.
- Yisehak K, **Becker A**, Belay D, Bosch G, Hendriks WH, Clauss M, Janssens GPJ (2011) Salivary amino acid concentrations in zebus (*Bos indicus*) and zebu hybrids (*Bos indicus* x *Bos taurus*) fed a tannin-rich diet. Belgian Journal of Zoology 141, 93-96.

BOOK CHAPTER

- Whitehouse-Tedd KM, Dierenfeld ES, **Becker AAMJ**, Huys G, Williams J, Depauw S, Kerr K, Janssens GPJ. Captive cheetah nutrition. In: Marker L, Schmidt-Kuentzel A, Boast L (Eds.) Conservation and Biology of the Cheetah. Chapter 28. Academix Press (Elsevier) (*in preparation*)

ORAL PRESENTATIONS

2015 Becker AAMJ, Microbial Life @ The Zoo: Intestinale Microbiota van de cheeta, invited speaker at Annual Meeting of the Nederlandse Vereniging van Dierentuinen (NVD) Voedingsgroep, Apenheul, Apeldoorn, The Netherlands

2015 Depauw S, Becker AAMJ, Animal Fibre: a key factor for gastrointestinal health in an obligate carnivore: the cheetah, 1st European Cheetah Workshop, Safaripark Beekse Bergen, Hilvarenbeek, The Netherlands

2014 Becker AAMJ, Microbial Life @ The Zoo, invited speaker at Centre for Research and Conservation (CRC) Zoo Research Symposium, Zoo Antwerp, Antwerp, Belgium

2014 Becker AAMJ, Digestive host microbiota of domestic cats and captive cheetahs: it's not all in the family, invited speaker at 14th American Academy of Veterinary Nutrition (AAVN) Research Symposium and American College of Veterinary Internal Medicine (ACVIM) Forum, Nashville, Tennessee, USA
supported by ESVCN/Waltham Student Award

2013 Becker AAMJ, Intestinal microbiota of domestic cats and captive cheetahs: worlds apart?, at 17th European Society of Veterinary and Comparative Nutrition (ESVCN) Congress, Ghent, Belgium
winner of the ESVCN/Waltham Student Award for best presentation

2012 Becker AAMJ, Microbial life at the zoo: the faecal microbiota of captive cheetahs, at 3rd International Workshop Gut Microbiota in Health and Disease, Maastricht, The Netherlands

POSTER PRESENTATIONS

2014 **Becker AAMJ**, Janssens GPJ, Hesta M, Huys G. Microbial life in strict carnivores: diversity and stability of the faecal core microbiota of captive cheetahs, at 2nd World Congress on Targeting Microbiota, Institut Pasteur, Paris, France

2012 **Becker AAMJ**, Hesta M, Janssens GPJ, Huys G. First insights into the gut microbiota of captive cheetahs, at 7th European Zoo Nutrition Conference (EZNC), Zürich, Switserland

2012 **Becker AAMJ**, Depauw S, Brinkman B, Hesta M, Janssens GPJ, Huys G. Monitoring bacterial population dynamics during *in vitro* fermentation of dietary animal substrates with cheetah faecal inoculum, at 14th Gut Day Symposium, Leuven, Belgium

2011 **Becker AAMJ**, Hesta M, Depauw S, Janssens GPJ, Huys G. Complexity and temporal stability of the digestive microbiota of captive cheetahs by community fingerprinting, at 15th ESVCN Congress, Zaragosa, Spain

2011 **Becker AAMJ**, Hesta M, Depauw S, Janssens GPJ, Huys G. Complexity and temporal stability of the digestive microbiota of captive cheetahs by community fingerprinting, at 13th Gut Day Symposium, Wageningen, The Netherlands

EDUCATIONAL SUPPORT

2010-2015 Assistant at several practical courses organized within the training in Biology and the training Biochemistry and Biotechnology at Ghent University (bachelor and master students)

2013-2014 Co-supervision of Niloufar Majdzadeh, Master thesis: Predominant faecal microbiota of captivve cheetahs housed in European zoos: a cross-sectional study (Master Biochemistry and Biotechnology, major Microbial Biotechnology, Faculty of Sciences, Ghent University)

2012-2013 Co-supervision of Annelies Tourny, Master thesis: Verkennend onderzoek naar de diversiteit en temporele stabiliteit van faecale microbiota bij cheeta's in gevangenschap (Master Biochemistry and Biotechnology, major Microbial Biotechnology, Faculty of Sciences, Ghent University)

2012-2013	Co-supervision of Amalia van Es, Master thesis: Wilde dieren in gevangenschap: mogelijke invloed van dieet op de intestinale microbiota (2nd year Master of Veterinary Sciences, Faculty of Veterinary Medicine, Ghent University)
2011-2013	Co-supervision of Charlotte Coppens, Wendy Beetens, Elke Stautemas and Kaatje Coppens (1st year Master Biochemistry and Biotechnology, Faculty of Sciences, Ghent University)

LANGUAGE SKILLS

DUTCH	Mother tongue
ENGLISH	Speaking: proficient user C1 | Writing: proficient user C2 | Understanding: proficient user C2
FRENCH	Speaking: independent user B2 | Writing: independent user B1 | Understanding: independent user B2
GERMAN	Speaking: independent user B1 | Writing: basic user A1 | Understanding: independent user B1
SPANISH	Speaking: basic user A1 | Writing: basic user A1 | Understanding: basic user A2

EXPERIENCE ABROAD

2008-2009	Scientific Research for Master Dissertation of Veterinary Sciences at Jimma, Ethiopia, framed within the Institutional Cooperation Project between Ghent University and Jimma University, supported by a VLIR Travel Grant (6 months)
2004	Voluntary Work at Conservation Centre Carapax, Massa Maritima, Italy, framed within the Flemish Voluntary group Bouworde (2 months)
2001	Music Exchange Project between Flemish and Canadian High Schools, Québec, Canada (1 month)

OTHER SKILLS & INTERESTS

ACTIVE AND PASSIONATE MUSICIAN	Piano Highest Grade 3 (2004, summa cum laude) Traverse Flute Highest Grade 3 (2004, summa cum laude) Cello Mid Grade 3 (in progress) 2009-... | Flautist at the Ghent University Student Wind Orchestra Band (GUHO)
SOCIAL AND ORGANIZATIONAL SKILLS	2009 | Co-Founder of the Ghent University Student Wind Orchestra Band (GUHO) (www.guho.be) 2009-2014 | GUHO Board member responsible for Public Relations and Publicity 2002-2009 | Board member and dancer in the art Group Mistral (www.mistralonline.be) 2012-2014 | Active participation in scientific popularization ('Dag van de Wetenschap') (www.dagvandewetenschap.be)

Dankwoord

'Above all, watch with glittering eyes the world around you, because the greatest secrets are always hidden in the most unlikely places. Those who don't believe in magic will never find it.' Roald Dahl

De finale.

In de muziek het laatste deel van een meerdelige compositie, veelal gekenmerkt door een uitbundig en feestelijk karakter. Het schrijven van een dankwoord is voor mij dan ook de finale van deze 'doctoraatscompositie'. Een finale rijkelijk gekleurd door de vele mensen die mij gesteund en aangemoedigd hebben.

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