The Gene Catalogue and Functional Analysis of the Gut Microbiome of Lions in Etosha National Park

by

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Declaration

I declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

(Signature of candidate)

5th day of September 2024 at 4 Sunninghill Road, Wynberg, Cape Town

Abstract

Characterising the microbiomes of free-living mammals may aid conservation efforts, yet the gut microbiome of carnivores is underrepresented. This study represents the first description of the gut microbiome of free-living African lions (Panthera leo melanochaita). Faecal samples from 20 lions were collected in Etosha National Park, Namibia and microbial DNA was extracted. Samples were then whole genome sequenced, and classified using MetaPhlAn and Genome Taxonomy Database toolkit. The two most abundant bacterial genera in the lions' gut microbiomes were Bacteroides (16.9%) and Phocaeicola (16.6%). Microbiome diversity was similar between the sexes and across seasons as assessed through Bray-Curtis dissimilarity and Shannon diversity index. The genus Clostridium_AH was more abundant in male lions ($P = 0.007$; d.f. = 22), while Aphodousia ($P = 0.003$; d.f. = 22) was more abundant in females. Lions captured in winter had a high abundance of Plesiomonas relative to those captured in summer ($P = 0.008$), whereas lions captured in summer a high abundance of Dysosmobacter ($P = 0.038$; d.f. = 22), Pelethomonas ($P = 0.021$; d.f. = 22), Metalachnospira ($P = 0.033$; d.f. = 22) and Clostridium Q ($P = 0.012$; d.f. = 22) compared to those captured in winter. Following various taxonomic classification approaches, a third of the reads (33.6%) present in the lion gut microbiome remained unclassified. We constructed 272 metagenome assembled genomes, from seven bacterial phyla, representing mostly new species which will contribute to understanding of the carnivore gut microbiome.

1. Introduction

Microbial communities in the gut are linked to key health factors such as diet and disease (Cho and Blaser, 2012; Kohl and Carey, 2016; McFall-Ngai et al., 2013; McKenney et al., 2018; Trevelline et al., 2019). The human (Almeida et al., 2019) and murine (Walter et al., 2020) gut microbiomes are arguably the most well understood of mammals. The microbiomes of wild animals' however, especially large carnivores, remain largely unexplored. Characterising microbiomes of free-living mammals, particularly those species of conservation concern, may aid conservation efforts for a few reasons. Firstly, the gut microbiome is closely related to global environmental change and health (Bahrndorff et al., 2016; Trevelline et al., 2019; West et al., 2019) and secondly, new microbiology tools are increasingly being integrated into conservation efforts, such as faecal microbiome transplants (FMTs) (Guo et al., 2020) and disease-marking bacterial species (Nkera-Gutabara et al., 2022).

1.1 Gut microbiomes and health

Animals have evolved alongside their gut microbiome for millions of years, and they rely on each other for essential nutrients and energy (Muegge et al., 2011; West et al., 2019). Probably the most well-known example of bacteria-host symbiosis is the fermentation of plant polysaccharides in the foregut and hindgut of herbivores (Dearing and Kohl, 2017; West et al., 2019). Carnivores also rely on their gut microbiomes for digestion and their gut microorganisms are specialised in polypeptide degradation (Muegge et al., 2011). The disruption of the gut microbiome can result in the inability to digest certain foods. For example, in woodrats (*Neotoma lepida*) inhibition of gut microbes via antibiotics prevents the digestion of the toxic creosote bush (*Larrea tridentate*), reducing their potential diet options and reducing survival rates (Kohl et al., 2014). Similarly, absence of the microbiome in the form of germ-free mice, rats, guinea pigs and rabbits retards growth compared to their conventionally raised counterparts (Crumeyrolle-Arias et al., 2014; Gordon and Pesti, 1971; Luczynski et al., 2016).

Not only do these microorganisms assist with the breakdown of food for their hosts, they can also create a barrier against disease by outcompeting pathogenic organisms (Round and Mazmanian, 2009) and regulating the innate immune system (Thaiss et al., 2016). A disruption of these bacteria is termed dysbiosis, briefly defined as "a breakdown in the balance between putative species of

‘protective’ versus ‘harmful’ intestinal bacteria” (Tamboli et al., 2004). Captive species are particularly vulnerable to dysbiosis as they often show lower diversity in their gut microbiome than their free-living counterparts, which may be caused by a change in diet (Ringø et al., 2016; Walker, 2017), high stress (Lavrinienko et al., 2020; Walker, 2017) or reduced interaction between parent and offspring (Hummel et al., 2022; Xie et al., 2016). Whatever the cause of dysbiosis, the increase in harmful bacteria might not only cause disease, but disease itself may also exacerbate the dysbiosis.

Microbial communities in the gut may change composition upon exposure to host pathogens (Peixoto et al., 2021; Qu et al., 2008). For example, chimpanzees (*Pan troglodytes*) infected with simian immunodeficiency virus (SIV) showed compositional changes in their gut microbiome linked to acquired immune deficiency syndrome (AIDS) progression (Barbian et al., 2018). Disease can even result in long-lasting changes to gut microbial composition, leading to susceptibility to re-infection. For example, the disease-causing protozoan *Cryptosporidium* causes a depletion in diversity of the gut microbiome of the Coquerel’s sifaka (*Propithecus coquereli*), even during recovery (Mckenney et al., 2017).

The complex interplay between health and microbiome composition across various host species is difficult to understand. Current research is aimed at uncovering the intricacies of these interactions, but the level of understanding varies greatly among different host species.

1.2 Microbiome of large carnivores

The gut microbiome of carnivores is not as well studied as herbivores and omnivores. To date, the focus of microbiome research has been on humans and animals of agricultural value such as cows, sheep and chickens. While carnivores are not of commercial concern, understanding microbiome functionality of free-living carnivores is of conservation concern. Carnivores play a fundamental role in maintaining ecosystem balance (Hoeks et al., 2020) and apex predators in particular are keystone species (Sergio et al., 2008).

The most abundant bacterial phyla in the gut of many carnivores are Bacillota / Firmicutes, Fusobacteriota and Bacteroidota in that order (de Jonge et al., 2022; Zhu et al., 2018). The carnivores with a high abundance of these phyla include spotted seals (*Phoca largha*), spotted hyena (*Crocuta crocuta*), Siberian tigers (*Panthera tigris altaica*), Bengal tigers (*Panthera tigris*

tigris), jaguar (*Panthera onca*) and foxes (*Vulpes vulpes*) (de Jonge et al., 2022; Zhu et al., 2018). Interestingly, strictly meat-eating carnivores uniquely show high proportions of Fusobacteriota compared to omnivores and herbivores, indicating that this phylum may be important to high protein and fat diets or that these diets may select for Fusobacteriota (Levin et al., 2021; Zhu et al., 2018). At a functional level, we know that carnivores carry a relatively high proportion of genes involved in uric acid degradation in their gut microbiome (Zhu et al., 2018). Favouring purine metabolism in the carnivore gut makes sense in the context of their high purine diet.

Although there is some research on the gut microbiome for the order Carnivora, papers on specific Carnivora species are limited. To date, only one paper has investigated the lion gut microbiome specifically (Mittal et al., 2020). This study found that the gut microbiome of three wild Indian lions (*Panthera leo*) differed from the gut microbiome of nine leopards (*Panthera pardus*) and nine tigers (*Panthera tigris*). Fusobacteriota was the most abundant phylum (35%) in lions, while tigers and leopards were consistent with many other carnivores, having Bacillota in the highest abundance (32% and 40%, respectively) (Mittal et al., 2020).

The most abundant genus in the three lions and nine leopards was *Fusobacterium*, a genus consisting of anaerobic, Gram-gram-negative bacteria often found on the mucous membrane of carnivores and omnivores, but rarely in herbivores (Hofstad, 1992; Levin et al., 2021). Certain *Fusobacterium* species are associated with disease in humans, such as *F. nucleatum* associated with colorectal cancer (Gethings-Behncke et al., 2020) and *F. necrophorum* associateed with Lemierre's Syndrome (Kristensen and Prag, 2000). However, another species, *F. varium* antagonises colonisation by the pathogens *Salmonella* and *Shigella* (Ushijima and Ozaki, 1986). Additionally, *Fusobacterium* species may serve a different function in other animals. For example, dogs fed a biologically appropriate, raw, meat-based diet have a higher abundance of *Fusobacterium* in their gut than dogs fed a less healthy, processed, dry food diet (Alessandri et al., 2020). It may be that *Fusobacterium* produce key proteolytic enzymes which aid protein digestion in carnivores. For example, *F. nucleatum* produces an endopeptidase known as fusolisin (Doron et al., 2014).

Currently, the best way to assess microbial functions in the gut is to characterise the host's microbiome. Microbiome characterisation can also help and quantify how well a given species is functioning in its environment, in terms of diet, stress, diversity and disease status is to characterise

its microbiome. A variety of tools are currently being added to the conservationist's toolbox to describe the gut microbiome.

1.3 The gut microbiome as a tool in conservation

In some cases, physical treatments such as faecal microbiome transplants (FMTs) (Guo et al., 2020; Ji et al., 2018) and probiotics (McKenzie et al., 2018) can be used to alter the gut microbiome. For example, kangaroo joeys (Osphranter rufus) in Alice Springs, Australia were cured of life-threatening diarrhoeal illness through FMT in the form of healthy kangaroo faecal pellets crushed in milk (Milliken, 2019). Studies using the Shannon Index have shown that gut microbiome diversity is reduced in species in fragmented and urbanised environments (Amato et al., 2013; Teyssier et al., 2018). This information can be useful to ecologists as they can monitor a community's microbial diversity to assess risk of disease. Additionally, steps can be taken to increase microbiome diversity in order to strengthen disease resistance. A straightforward step would be to facilitate interactions between individuals from different communities to allow for transfer of microorganisms. For example, green frogs (Lithobates clamitans) have been inoculated against harmful fungal infections by horizontal transfer of an antifungal skin bacterium from one frog to the environment (in this case through water transmission) to another host (Rebollar et al., 2016). However, physical interventions are uncommon because they can be more detrimental than beneficial, especially when the microbiome associated with hosts is not understood for most species (Dallas and Warne, 2023). Creating these 'catalogues' or 'libraries' is the first step to assessing the need for FMTs.

1.3.1 Diversity and phenotype-linked bacterial species

Gut microbiome catalogues or libraries are often presented as percentage compositions of different bacterial, fungal or viral taxa associated with the gut. As our understanding of microbial species grows, knowledge of their abundances can help ecologists draw connections between the microbes' role and the host's physiology. For example, a high abundance of pathogenic microorganisms across a host species, could indicate host resistance to that microorganisms' disease-causing mechanisms of these pathogens (Speight et al., 2019). Similarly, host species with a similar abundance of the same microbial species in their gut microbiome indicates a similar diet or behaviour, for example, herbivores share a largely conserved gut microbiome compared to carnivores (Zhu et al., 2018).

The gut microbiome is useful as a tool to monitor biodiversity. Two metrics for diversity are: alpha diversity, diversity within a community, and beta diversity, diversity between communities (Spellerberg and Fedor, 2003). Alpha diversity is the diversity within a sample, ecosystem or site (Willis, 2019). For example, an individual with many species of bacteria in their microbiome has a higher alpha diversity than an individual with one species. Beta diversity, on the other hand, is a measure of similarity between different samples, ecosystems or sites (Whittaker, 1960). For example, a community comprising two individuals with vastly different microbiome compositions would have a higher beta diversity compared to a community comprising two individuals with the exact same microbiome composition.

There are multiple ways to compute each of these metrics. Alpha diversity can be measured in a simple way, for example using the Shannon index by counting the numbers of species observed in a sample (Xia and Sun, 2023), where samples with more species have a higher abundance than those with fewer. However, calculations are frequently inaccurate when classification mechanisms use incomplete databases and species that cannot be identified are excluded from calculations. Additionally, some samples may have a high number of species but these species may all be closely related. It is therefore important to weight species based on their relatedness (Kers and Saccenti, 2022). For example, some alpha diversity measurements quantify phylogenetic differences and incorporate these rather than using only species presence/absence (Bolyen et al., 2019). This avenue encounters similar problems related to database incompleteness; a clade of phylogenetically related, well-documented species are more likely to show up in samples since they are well categorised, the inverse being true for understudied species.

Beta diversity similarly encompasses various metrics. One common approach to measuring beta diversity uses species abundance profiles among samples through metrics such as Bray-Curtis dissimilarity or Jaccard distance, which quantify the extent of overlap or dissimilarity in species composition between samples (Kers and Saccenti, 2022). However, as with alpha diversity, beta diversity measurements may encounter challenges with incomplete databases. Consequently, database agnostic approaches, such as *k*-mer analysis using Sourmash, offer an alternative means to assess beta diversity. *K*-mer analysis divides reads into smaller *k*-mers of a set nucleotide length and compares sequence overlap between *k*-mers rather than using taxonomic annotations, thus removing the biases of classification-based approaches (Irber et al., 2022). Reference agnostic

tools can have their own challenges as they do not take into consideration any functional or morphological differences and similarities between genomes or genes. Using a reference-based and reference-agnostic approach to assess beta diversity can be beneficial as the outputs of both can be compared (Ponsero et al., 2023). Linear regression modelling can be used on distance matrices to assess differences in beta diversity while also taking into account potentially confounding variables (Legendre, 2008).

1.3.2 Sequencing methods

To obtain abundances of gut microbiome taxa, DNA must be extracted and sequenced from microbiome samples. Since the advent of next-generation sequencing technologies, various sequencing methods have been developed to analyse microbial communities. One widely used approach to obtain gut microbiome data is targeted next-generation amplicon sequencing of the 16S ribosomal ribonucleic acid (rRNA) gene (Ranjan et al., 2016). This sequencing technique is commonly employed because it is well-studied and relatively affordable. (Bahrndorff et al., 2016; Dave et al., 2012).

The 16S rRNA gene contains highly conserved regions that flank several hypervariable regions present in members of the domains Bacteria and Archaea (Wasimuddin et al., 2017). Primers designed to target these conserved regions are used in a polymerase chain reaction (PCR) to amplify the 16S rRNA gene from the DNA extracted from the microbiome samples. (Wasimuddin et al., 2017). This amplification step ensures that sufficient quantities of the target genes are available for sequencing. The hypervariable regions of the 16S rRNA gene allow for the differentiation and classification of bacterial and archaeal species.

The reads generated from 16S rRNA amplification and sequencing are then compared to reference databases to create operational taxonomic units (OTUs). Operational taxonomic units are clusters of similar 16S rRNA sequences that are grouped based on a specified level of sequence similarity, typically 97% or 99% (Chen et al., 2013). . These OTUs can then be assigned to taxa, allowing identification of the abundance and presence of microorganisms (Zhu et al., 2018).

Despite the valuable information provided by targeted next generation sequencing of the 16S rRNA gene, this technique does not provide data on the functional roles of the microorganisms present. A technique which can fill in these gaps is whole metagenome shotgun sequencing. Shotgun metagenome sequencing sequences the entire genomes of all microorganisms present in the sample, allowing for identification of bacteria, archaea and eukaryotes (Wasimuddin et al., 2017). This approach identifies rare species and the metabolic functions of microorganisms (Wasimuddin et al., 2017). The drawbacks of whole genome sequencing are the higher costs compared to targeted next generation sequencing and the higher risk of contamination with host DNA (Wasimuddin et al., 2017). Shotgun sequencing is especially helpful in the context of the host species because scientists can establish what functions gut microorganisms carry out for the host. For example, the gut microbiome of carnivores has a relatively high proportion of genes involved in uric acid degradation (Bauer et al., 2016), assisting the host with breakdown of proteins and fats. Both targeted next generation sequencing and shotgun metagenome sequencing yield DNA sequences which are not ecologically helpful in and of themselves. The fragments of DNA generate sequences of base pairs known as DNA reads. These reads need to be classified in order to confirm the presence and abundance of microorganisms for biological interpretation.

1.3.3 Classification tools

Classifying reads to a deep taxonomic level in microbiome samples provides insights into microbial community composition and function which can be useful from an ecological perspective.There are many tools used to answer the question of what microorganisms are present in a microbiome sample. Classifying reads in metagenomic samples to the species, genus and even phylum level presents a number of hurdles. Many species present in microbiome samples have never been sequenced. Known species are represented in databases. Databases are created by collating genomes which are generated from reads from previously sequenced environments. Many unknown species can at least be categorised based on their phylogenetic proximity to known species. However, many microorganisms exhibit high sequence similarity, posing difficulties in distinguishing their presence and absence within a sample.

To address these complexities, a plethora of sequence alignment tools have been developed specifically for the classification of reads. These tools normally employ one of two methods: large database tools classify all sequences with varying levels of confidence while marker gene tools

classify only high confidence reads, leaving sequences unclassified if insufficient evidence or alignment exists. For example, if a read lines up to three or four genomes, marker gene tools would rather leave it unclassified while a large database tool would assign the read to the genome with the highest alignment. In addition to large database and marker gene classification models, k-mer classification has been featured in more recent studies (Van Etten et al., 2023).

Perhaps the most well-known large database tool is the Basic Local Alignment Search Tool (BLAST) program (Wheeler and Bhagwat, 2007). BLAST breaks down query sequences into *k*-mers based on the word count specified by the user and compares these *k*-mers to a database, giving scores based on nucleotide or amino acid similarity and outputting the most similar classification. BLAST itself was not designed for metagenomic classifications; however, a number of programs have repurposed the BLAST alignment algorithm for these large datasets. For example, MEtaGenome ANalyzer (MEGAN) performs BLAST comparisons of query sequences and provides an ordered summary of identified taxa (Huson et al., 2007). Similar to MEGAN, Phymm plus BLAST (PhymmBL) is a tool that combines BLAST results with computer learning to improve accuracy and classify short-length reads (Brady and Salzberg, 2009). Unlike MEGAN and PhymmBL, Kraken2 splits reads into k-mers and searches for exact-match queries in a database rather than scoring them (Wood and Salzberg, 2014). Kraken2 then scores reads based on the number of exact-aligned k-mers in a taxonomy tree. The *nt* (nucleotide) database is a comprehensive collection of nucleotide sequences, spanning multiple kingdoms and domains including bacteria, archaea, fungi, plants, and animals. However, the extensive size of this database requires significant computing power and storage capacity, making it resources and time intensive.

These tools all face the challenge of distinguishing between phylogenetically similar taxa. For example, species from the genus *Bacteroides* have approximately 97.5% nucleotide similarity in several genes as well as the 16S rRNA gene (Sakamoto and Ohkuma, 2011). This high sequence similarity means that if fragments from these genes are present in a sample, it would be near impossible to distinguish to which species these genes correspond. In addition, programs like BLAST require substantial computational resources and have prolonged processing times.

To overcome these hurdles, abundance estimation programs such as MetaPhlAn (Blanco-Míguez et al., 2023), mOTUs (Milanese et al., 2019) and GTDB-Tk (Chaumeil et al., 2022) have been created. These programs use smaller databases containing ‘marker genes’ specific to certain taxa

and estimate abundance of taxa based on sequence alignment to the marker genes. These database methods classify taxa more accurately and in a shorter time than BLAST. However, using a marker gene approach creates its own set of challenges; marker genes ignore a large number of reads present in metagenomic samples. Using only marker genes is especially problematic for samples from unique or under researched sources which are likely to contain species not present in marker gene databases at all. It is estimated that 40 to 50% of microbial species do not have a reference genome (Nayfach et al., 2016). The inability to fully explore microbiome samples is slowly being remedied as databases are added to using microbial genomes created from sequencing samples from new environments.

1.3.4 MAG Creation

Entire bacterial genomes are a rich source of information for microbiologists since they contain a bacterium's entire suite of genes, regulatory elements and non-coding regions. Traditionally, microbial genome sequencing entailed the prior culturing of the bacterium on artificial media. However, it is estimated that close to 99% of prokaryotes cannot be cultured, making it impossible to sequence their genomes in this way (Rinke et al., 2013). Metagenome-assembled genomes (MAGs) provide a new way of creating and cataloguing bacterial genomes. MAGs are created by assessing overlapping regions of fragmented reads created from metagenomic sequences and creating longer nucleotide sequences by assembling these reads.

The creation of MAGs is typically performed in one of two ways. Either by individual assembly of MAGs from each sample or by co-assembly of MAGs using reads from all samples. Research into the two approaches has shown pros and cons to both (Delgado and Andersson, 2022). In brief the co-assembly approach is useful for small sample sizes and for identifying rare genomes which occur across multiple individuals of the host species. On the other hand, the individual assembly approach is more applicable for finding specific microbial species strains which exist in only a few of the host species (Royo-Llonch et al., 2021).

Two programs designed specifically for assembly of metagenomic reads are MEGAHIT (Li et al., 2016) and MetaSPades (Nurk et al., 2017). Both tools use similar assembly algorithms; however MEGAHIT usually has lower memory requirements and focuses on short-read assembly compared

to MetaSPades, which is more specialised to hybrid assemblies for addition of long-read sequences like Oxford Nanopore Technology (ONT) reads.

After reads are assembled into contiguous sequences or contigs, they can be binned into MAGs. Binning is the process of grouping similar contigs into 'bins' and assigning these groups to representative genomes known as MAGs. Similarity can either be decided based on sequence similarity, for example average nucleotide identity (ANI) or using compositional features like GC content or biassed use of a certain codon.

Three common tools exist for the binning process: MetaBat, MaxBin and Concoct. MetaBat operates by employing an algorithm that considers factors such as tetranucleotide frequency, contig abundance, and coverage across various samples to group contigs into MAGs (Kang et al., 2019). MaxBin similarly measures tetranucleotide frequencies of contigs but also their coverages for all metagenomes and uses probability values to assign them to bins (Wu et al., 2016). In contrast, Concoct measures contig coverage across samples to bin into MAGs (Alneberg et al., 2014). Overall there is no need to exclude any of these tools since the outputs can be combined and de-replicated using a complementary program, DAStool (Sieber et al., 2018). Combining the binning tools integrates their respective advantages while mitigating disadvantages by compensating for each other's limitations. Over and above their addition to databases, MAGs have a further layer of usefulness; all of the genes in a species are present in the created MAG and the proteins created by these genes can be analysed to identify pathways and functions carried out by that species.

1.3.5 Functional analysis

Functional analysis is used to identify potential metabolic roles of microbial communities and is important for unravelling host-microbe interactions, which play a pivotal role in the health and ecology of organisms. Functional analysis typically begins with the prediction of protein-coding genes from DNA sequences. This step involves identifying open reading frames (ORFs) within the DNA sequences, which are regions that potentially encode proteins. Tools such as Prodigal (Hyatt et al., 2010) and MetaGeneMark 2 (Gemayel et al., 2022) are commonly used for gene prediction. Both Prodigal and MetaGeneMark 2 use prediction algorithms known as Hidden Markov Models (HMMs). Briefly, Markov models use a statistical model in which the probability

of an event is predicated on the previous state of the model and is not affected by any state of the model before that (Eddy 1996). For example, a Markov model can be created for a sequence of DNA where the probability of a nucleotide is based upon the identity of the previous nucleotide in the sequence. These probabilities in each model will differ with different genes or gene properties. For example, CpG islands will have a higher probability that a cytosine nucleotide is followed by a guanine nucleotide compared to most other genes. This property can be used to identify the probability that a certain sequence has a certain property. Put another way, given a sequence of observations (in this case nucleotides) the HMM is used to find the most likely sequence of hidden states (in this case gene properties). (Mor et al., 2021). This technique allows for inference of gene functions and the biological processes encoded in microbial genomes present in the samples.

Once protein-coding genes are predicted, the next step is to assign these proteins to known biological pathways or functional categories. This process involves mapping the predicted proteins to reference databases such as the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa, 2002). Tools like KEGG Mapper (Kanehisa and Sato, 2020), GHOSTkoala (Kanehisa et al., 2016) and Clusters of Orthologous Groups (COG) Database (Galperin et al., 2021) can be utilised for this mapping step. These tools compare the predicted proteins against curated databases of annotated genes and proteins, assigning functional annotations based on sequence similarity or orthologous relationships. KEGG Mapper is a more comprehensive program with a variety of use-specified tools while GHOSTKoala is a relatively simple web-based tool. The COG database on the other hand offers a more generalised classification scheme, encompassing a wide range of biological functions and processes across diverse organisms. Despite their significance, the majority of host-microbe interactions remain elusive, particularly within diverse and relatively understudied taxa such as the order Carnivora.

1.4 Research questions and objectives

Despite the vital role of the gut microbiome in carnivore health and ecology, there remains a notable scarcity of species-specific data, with most studies aggregating carnivores together (Wu et al., 2022; Youngblut et al., 2020; Zhu et al., 2018). Furthermore, existing research on the lion gut microbiome is notably sparse, with only one published study examining a small sample of Asiatic lions (*Panthera leo leo*) rescued from a circus in India. By expanding upon this limited body of literature, this research seeks to provide a comprehensive analysis of the gut microbiome in free-

living lions. This endeavour is particularly pertinent as it represents the first microbiome analysis conducted on African lions (*Panthera leo melanochaita*). A strength of the project is that the lions were sampled in their natural habitat.

Therefore, the aim of this study was to create a 'core' library and identify metabolic functions of the gut microbiome of free-living lions. To achieve this aim, the objectives of the dissertation are to::

1. Create a core catalogue of the most abundant microbial species and genera in the lion gut.
2. Determine whether sex and season are associated with significant variability in the composition and diversity of the lion gut microbiome.
3. Identify and characterise novel prokaryotic species in the lion gut microbiome through creation of MAGs and comparison of these MAGs to current databases.
4. Infer important metabolic functions performed or facilitated by microorganisms in the lion gut microbiome.

2. Methods

2.1 Study site and species

Faecal samples were collected from free-living lions in Etosha National Park (Figure 1). The park is a 22 270 km^2 fenced reserve in northern Namibia. Etosha is subdivided into three major habitats; woodlands on the far west and east of the park, open grassland plains in the centre and a hypersaline pan in the central region (Heydinger and Packer, 2022). The park experiences approximately 350-460mm of rainfall per year (Turner et al., 2022), with December to March being the hottest and wettest months (Turner et al., 2022). In winter, temperatures range from 18-28°C during the day, while below zero temperatures are common at night. In the summer, temperatures range from 20-34°C during the day (Turner et al., 2022). The sampled lions primarily occupied the plains area surrounding Okaukuejo (19.175°S, 15.924°E).

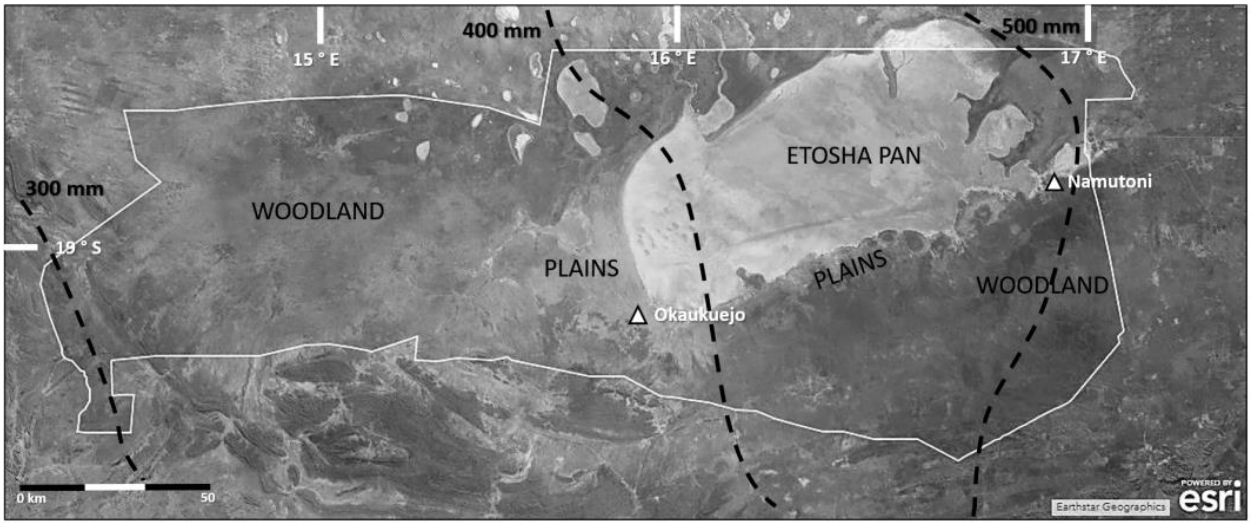

Figure 1: Etosha National Park major habitats. White line represents the border of the Park. Dashed, black lines represent rainfall isohyets. The triangle marks Okaukuejo camp. Image taken from (Heydinger and Packer, 2022).

Lions are registered as vulnerable by the International Union for the Conservation of Nature (IUCN) Red List41 and across West, Central and East Africa, their populations have dropped by 43% over the last 21 years (Bauer et al., 2016, 2015). Populations from these areas are projected to drop by a total of 50% within the next two decades (Bauer et al., 2016, 2015). Conversely, the lion population in southern Africa has increased by about 12% over the same 21 years (Bauer et al., 2016, 2015).

Etosha National Park in Namibia contains an estimated 500 lions (Heydinger and Packer, 2022). Lions in this park commonly feed on gemsbok (*Oryx gazelle*), wildebeest (*Connochaetes taurinus*), plains zebra (*Equus quagga*), springbok (*Antidorcas marsupialis*), giraffe (*Giraffa camelopardalis*) and warthog (*Phacochoerus africanus*) (Berry, 1981; Heydinger and Packer, 2022). The ungulate prey themselves survive on the ubiquitous perennial short-grasses, except for the browsing giraffe (Heydinger and Packer, 2022).

2.2 Sample collection

This microbiome project was part of a larger carnivore project in a collaboration of the University of the Witwatersrand with the Leibniz Institute for Zoo and Wildlife Research and the Etosha Ecological Institute. The study lions were sedated for the purpose of fitting or removing tracking collars and collecting health status information, and fresh faecal samples. A total of 23 faecal samples were collected from 20 lions, across two sampling sessions (Table 1)

Table 1: Demographic data for the 20 immobilised lions. Summary of the number of faecal samples collected from lions in Etosha National Park, Namibia.

	winter (May)[#]	summer (October)[#]	Total
	11	12	20
Sex			
Male	5	3	6
Female	6	9	14
Age			

3-4 years	2	1	3
5-8 years	4	7	10
9-10 years	2	2	3
Unknown	3	2	4

[#]Three lions were sampled in both May and October. The winter and summer columns show the number of samples collected while the total column shows the number of lions sampled.

The samples consisted of microbiome DNA extracted from 20 lions (males and females) distributed across 12 prides. The larger study targeted adult lions, with samples collected from lions across an age range of 3 to 10 years old. Immobilisation of the lions took place in two seasons, namely winter (May 2022) and summer (October 2022), allowing for repeated sampling to assess temporal changes in the microbiome of three individuals.

Lions were immobilised by a veterinarian from a vehicle. The dart contained a combination of 8-10 mg Medetomidine (Novecy Compounding Pharmacy, Namibia: Medetomodine 20 mg/mL) and 80-100 mg Zoletil 100™ (Virbac South Africa: 250 mg Tiletamine, 250 mg Zolazepam as dry powder reconstituted to 200 mg/mL). While the lion was immobilised, fresh faecal samples were collected (see below) and individual data on age, sex, pregnancy status, date and pride composition, were recorded where possible. Following sample collection and collar fitment or removal, the anaesthetic was reversed with 40-60 mg Atipamezole (Atipamezole 50 mg/mL; Novecy Compounding Pharmacy, Namibia) and 37.5 mg Yohimbine (Yohimbine 6.25 mg/mL; Novecy Compounding Pharmacy, Namibia). Lions were mobile within 5 to 10 minutes and rejoined their prides within an hour.

A clean spatula was used to scrape 1-5 ml of fresh faecal sample into OMNIgen GUT tubes (DNAGenotek, Kanata, Canada). Once the cap was full, the tube was closed and shaken to mix the sample with preservation liquid (see Figure 2 for details). The samples were placed in a cooler box at ~15°C within 5 minutes of closing the cap and then moved to a −20°C freezer within 24 hours for longer term storage

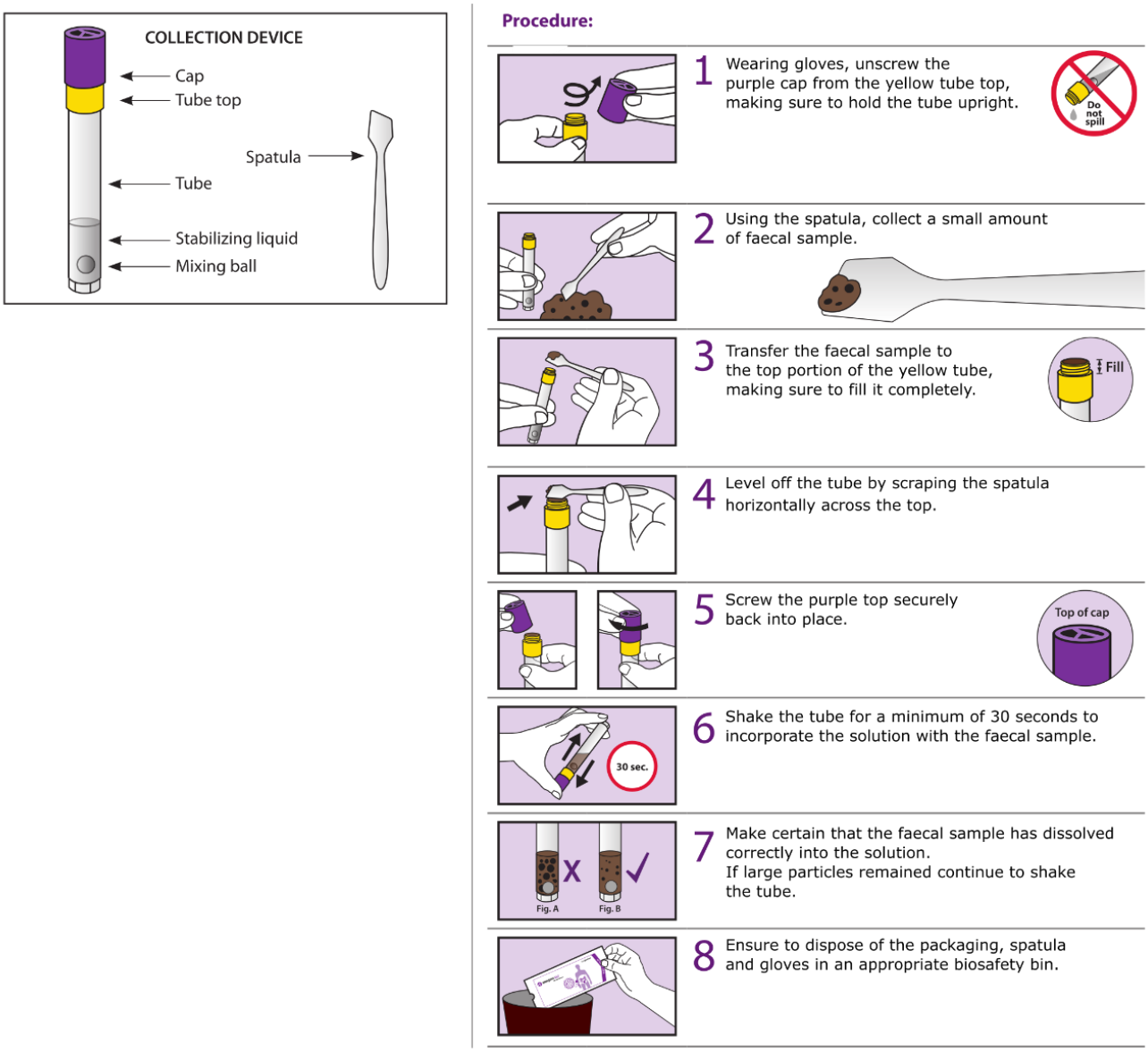

Figure 2: Procedure for collecting lion faecal samples using OMNIgene GUT tubes. This figure was adapted from the DNA Genotek manual, the full version of which can be found at https://www.dnagenotek.com/row/support/collection-instructions/omnigene-gut/OM-200.html

Permission for the research was obtained from the National Commission on Research, Science and Technology (NCRST) non-Namibian based research permit number RPIV01052020. Ethical clearance for the larger lion project was obtained through the University of the Witwatersrand Animal Ethical Screening Committee (AESC 2019/04/24C), with a modification added to include the collection of faecal samples for microbiome analysis. The CITES permits were obtained for export of samples from Namibia and import into South Africa, permit numbers 142695 135348. Veterinary clearance (import permit for pathology specimens number 13/1/1/30/2/202306000494) and a section 20 permit for disease control (permit number 24C) were obtained. The process of getting regulatory approval from both Namibia and SA for shipment of material was complex — a document describing the process of acquiring these permits and giving advice for future researchers can be found in the appendix section 8.1.

2.3 Sample transport and storage

The remote nature of Etosha National Park provided a challenge for transport and storage of samples. The samples were put into preservation liquid within 2 minutes of collection. The DNAGenotek tubes contain a preservation liquid designed to keep DNA stable for up to one year at −20°C (DNAGenotek, 2022; Doukhanine et al., 2016). Once in preservation liquid, samples can withstand 24 hours at room temperature without adverse effects to the quality of bacterial DNA in preservation liquid (Wasimuddin et al., 2017). Manufacturer instructions state that the DNA can remain stable for up to 60 days at ambient temperature (DNAGenotek, 2022). However, the nearby research centre in Ongava Game Reserve facilitated storage of the samples in a -20°C freezer. Ongava Research Centre is approximately a 30 minute drive from collection sites in Etosha National Park and samples were transported to Ongava Research Centre (ORC) about 15 km away within 24 hours of collection. Samples were frozen there for a maximum of six months before DNA extraction. While the preservation liquid allows for multiple freeze-thaw cycles without significant change to shotgun sequencing output (Doukhanine et al., 2016), freeze-thaw cycles were kept to a minimum and the lion samples were only thawed once prior to DNA extraction. Once extracted, the DNA was stored in a -20°C freezer.

2.4 DNA extraction and sequencing

After the final samples were collected in October 2022, genomic DNA was extracted from each sample from 250 milligrams of faecal sample using the DNEasy PowerSoil extraction kit (QIAGEN, Hilden, Germany). The manufacturer protocol was followed.

Preparation consisted of: wiping down the workspace with a 10% bleach solution to kill any microorganisms and remove contaminant DNA; rinsing the bleach off with distilled water; spraying 70% ethanol on the surfaces as a final disinfectant ; and heating a high salt solution (solution CD3) at 60°C in a heat block to dissolve the precipitate.

For each sample, 250 mg preserved faecal samples was added to a bead-beating tube (PowerBead Pro Tube) containing plastic beads that lysed the microbial cells when shaken in a TissueLyser. Each PowerBead Pro Tube was centrifuged for 5-10 seconds to ensure beads settled at the bottom. Then 250 mg of sample was pipetted into a collection tube on a scale accurate to 0.01 mg. After confirming the mass, the sample was added to the PowerBead Pro Tubes along with 800 µl of a buffer (Solution CD1) that helped to remove soil particles, dissolve humic acids and protect nucleic acids from degradation. The solution was then incubated in a heat block at 65°C for 10 min to ensure that the buffer homogenised properly with the sample. After incubation, the PowerBead Pro Tube was placed in a Tissuelyser at 25 Hz speed for 10 min and then centrifuged at 15,000 x g for 1 min. The Tissuelyser shakes the tubes, ensuring that bacterial cell walls are broken open and DNA is released into the buffer.

Further lysis was performed using chemical methods; 500-600 µl of the supernatant was transferred to a 2 ml microcentrifuge tube and 200 µl of solution containing inhibitor removal technology (Solution CD2) was added and the solution was vortexed for 5 seconds. Solution CD2 precipitated out the cell debris, humic acids and proteins, while leaving the DNA in solution. The microcentrifuge tube was centrifuged at 15000 x g for 1 min to create a pellet made up of the precipitated contaminants while the DNA remained in solution.

Total genomic DNA was then captured on a silica membrane while impurities were washed through. This was done by transferring 700 µl of the supernatant (containing DNA) to a new 2ml microcentrifuge tube, adding 600 µl of a high salt solution (Solution CD3) and vortexing the tube for 5 seconds. DNA binds with silica at high salt concentrations, allowing contaminants still

present at low levels to wash through the column. Next, 650 µl of the lysate was loaded onto a tube with a silica membrane at the bottom rested inside a collection tube (altogether called a membrane (MB) Spin Column) and centrifuged at 15 000 x g for 1 min. The flow through containing impurities was discarded and the centrifugation was repeated with another 650 µl of lysate to ensure that all the lysate had passed through the filter and as much DNA as possible was captured in the silica membrane. The filter part was carefully removed from the MB Spin Column and placed into a clean 2 ml collection tube. Then, 500 µl of wash buffer (Solution EA) was added to the MB Spin Column and centrifuged at 15 000 x g for 1 min. Solution EA washes away proteins and other undissolved contaminants from the spin column. The flow-through was discarded and the MB Spin Column placed back into the same 2 ml collection tube. Further impurities were removed by adding 500 µl of ethanol-based wash solution (Solution C5) and centrifuging the MB Spin Column at 15 000 x g for 1 min. Solution C5 washed away any remaining salts, acids and other contaminants. The flow-through was discarded and the MB Spin Column carefully placed into a new 2 ml collection tube. The MB Spin Column was centrifuged at 16,000 x g for 2 min to ensure all of the flow through was washed out.

DNA was then eluted from the membrane using a wash buffer. To do this, the MB Spin Column was placed into a 1.5 ml elution tube and 50 µl of 10 mM Tris buffer (Solution C6) was added to the centre of the white filter membrane. Solution C6 has low salt concentration and so the DNA which was previously bound to the silica membrane at high salt concentrations was released. The MB Spin Column was centrifuged at 15 000 x g for 1 min. Then another 50 µl of Solution C6 was added to the tube and centrifuged again to elute the remaining DNA. Finally, the MB Spin Column was discarded and the DNA present in the 1.5 ml Elution Tube was stored. DNA from each sample was extracted in duplicate with an average final elution of 60 µl and stored at -20°C.

Following acquisition of the necessary permits (see section 2.1 Sample collection), DNA was shipped in a temperature controlled box via air freight to the Sydney Brenner Institute for Molecular Biology (SBIMB) in Johannesburg, South Africa. The total time between freezers was 36 hours. Samples were frozen again for 24 hours. The samples were thawed and DNA concentrations were measured using a Nanodrop One machine (ThermoFisher Scientific Nanodrop One). Sample DNA concentration ranged from 1 to 48 ng/µl. Specific DNA concentrations for each sample can be found in appendix section 8.2.

Because four samples did not meet the recommended 10 ng/µl concentration required for shotgun sequencing, ethanol precipitation was attempted in order to increase the concentration of the DNA of two test samples that had duplicate extractions (Sperling et al., 2018). In the first step of ethanol precipitation the DNA solution was mixed with 4 µl of 5M NaCl. The NaCl ions neutralised the DNA, making it soluble for the next step. Then 200 µl of 100% ethanol was added to dissolve the DNA into a nonpolar solution, causing precipitation. After vortexing for 10 seconds to ensure precipitation, the samples were centrifuged at 10,000 x g for 5 minutes, causing the precipitated DNA to collect in the pellet. The ethanol was allowed to air dry for 15 minutes before resuspending the DNA in 20 µl of sterile water and measuring the DNA concentration using a Nanodrop One. However DNA concentrations did not increase. This process was attempted again four more times with four more duplicate samples, changing a few steps to try and concentrate the DNA. Firstly, the samples were placed in a -20°C freezer for 5 minutes after addition of the NaCl solution to improve DNA precipitation. On the next sample the previous freezer step was repeated and the sample left to air dry for 30 minutes instead of 15 minutes after centrifugation. On the third sample, the centrifugation time was increased from 5 minutes to 15 minutes to ensure the pellet formed. Finally, the NaCl concentration was increased from 5M to 10M. Unfortunately, none of these additions resulted in an increase in the DNA concentration. Nevertheless, one extraction was sent from all samples to the National Institute for Communicable Diseases (NICD) sequencing core facility for shotgun sequencing. Although four of the samples were sent with a concentration below 10 ng/µl (F2, F9 and M8), at least 100 ng of DNA was sent for every sample. The sample DNA concentrations were measured again by the NICD using a Qubit and Tapestation. DNA preparation was performed using the Illumina DNA prep kit and PCR was performed using the Illumina DNA prep reference guide ("Illumina DNA Prep Reference Guide"). The samples were sequenced on the Illumina Nextseq 2000 platform.

2.5 Data analysis

The bhattlab/AWIGen2Microbome pipeline (Figure 3) was used for processing of metagenome sequencing for two reasons. The first reason was pragmatic, as it used multiple classification tools, allowing a choice for what best suited the input of the following processes. Secondly, it integrated multiple binning tools, MetaBAT2, MaxBin2, and CONCOCT, and collated these using DAStool, combining the strengths and reducing the weaknesses of each tool.

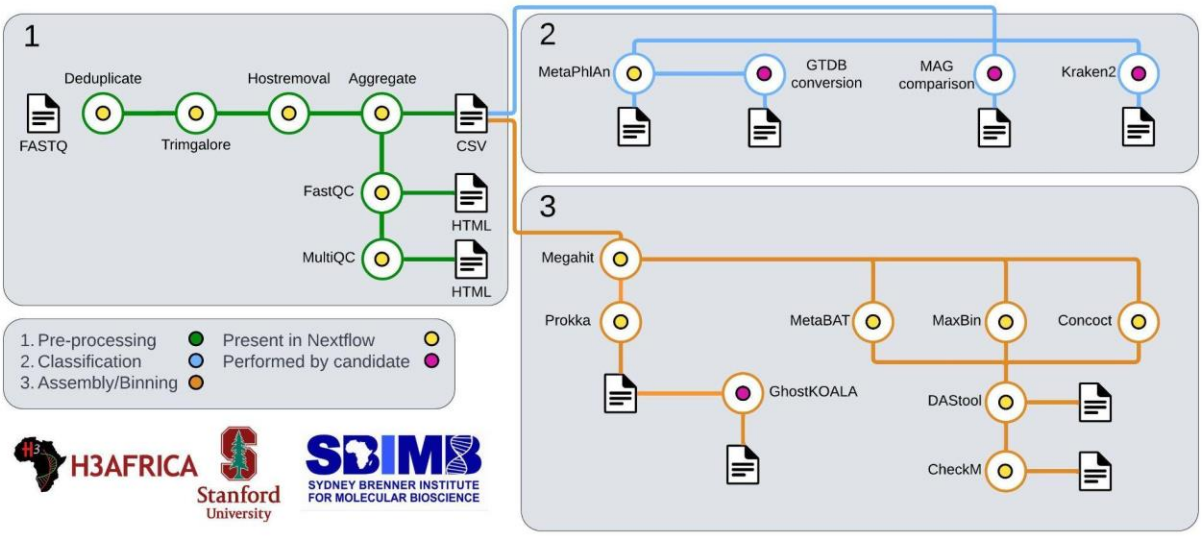

Figure 3: Nextflow pipeline used to process DNA reads created from the gut microbiome of lions in Etosha National Park, Namibia. Box 1 shows the steps for pre-processing the samples for removal of low quality reads and quality report generation. Box 2 shows the steps used for taxonomically classifying the reads. Box 3 shows the steps for assembling the reads into contigs and binning them into groups.
GTDB = genome taxonomy database; MAG = meta-genome assembled genome

Five samples from two previous research projects were included in the Nextflow pipeline analysis as references. Two samples were from lions darted in South Africa in 2014 (Youngblut et al., 2019), the samples from this study were sequenced using 16S amplicon sequencing. These samples came from a study which sequenced the genomes of fish, birds and other mammals, including lions; however the gut microbiome of the lions was not discussed specifically. The remaining three were from lions rescued from a circus in India, collected and sequenced in 2017 (Mittal et al., 2020). After processing and sequencing the lion samples collected in South Africa were excluded. Firstly, these samples were sequenced using 16S amplicon sequencing, while the pipeline used in this study is designed for whole genome sequence classification. Secondly, the samples from South African lions were sequenced 3 years prior to the samples from lions in India and 8 years prior to this study. The older extraction and sequencing technology likely created a notably different output compared to more modern technologies. Technological advancements and methodological refinements over the years have markedly enhanced the reliability and accuracy of sequencing outcomes since early 2010 (Zhou et al., 2022). Additionally, the classification of these samples was considerably different from both the lions in this study and the reference lions sampled in

India, skewing averages for the reference samples. The discrepancies in classification along with the older sequencing technology raised concerns about the reliability of these samples and were thus enough reason to exclude them. MetaPhlAn classifications for these samples can be found in appendix section 8.4.

2.5.1 Pre-processing raw reads

All reads (including South African and Indian reference samples) underwent pre-processing which involved removal of duplicate and low quality reads. Trimgalore parameters were set to a read quality (Phred score) above 30 and minimum read length of 60 base pairs. Lion sequences were also removed from the samples by aligning them to a lion reference genome from Ensembl using Burrows-Wheeler Aligner Maximal Exact Matches (BWA MEM) (Li and Durbin, 2009). BWA MEM dynamically applies a quality score to sequence alignments based on read length, nucleotide matches and mismatches and gap lengths (Li and Durbin, 2009). The quality of reads were then assessed using FastQC and MultiQC (Ewels et al., 2016).

MultiQC analysis revealed that the percentage of duplicate reads was low, between 11% and 30% with an average of 19.6%, indicating effective removal of redundant sequences. The percentage of GC content was within the expected range of 40-50% (Ely, 2021), which is important for ensuring unbiased representation of the genome. The average read length was 146 base pairs, aligning well with the 150 base pair read length recommended by Illumina (Huptas et al., 2016). The MultiQC results for forward and reverse reads can be found in section 8.3 of the appendix.

2.5.2 Taxonomic classification of reads

Pre-processed reads were taxonomically classified using three tools: MetaPhlAn 4.0.6 (Blanco-Míguez et al., 2023) GTDB-tk (Milanese et al., 2019) and Kraken2 (Wood et al., 2019). MetaPhlAn 4 uses 4 992 new species-level genome bins (SGBs) implemented in 2023, created from metagenome assemblies and isolate genomes which explain more reads from low-characterised environments such as the lion gut microbiome. For this reason MetaPhlAn was chosen over mOTUs as the first classification output. The MetaPhlAn abundance output was converted to Genome Taxonomy DataBase (GTDB) taxonomy using a new script created by the MetaPhlAn developers. The use of GTDB taxonomy helps with interpretation of results as little to no literature is available on the origin or functionality of species level genome bins (SGBs). On the other hand,

GTDB provides a comprehensive database with representative species and origins of genomes. For example, SGB1818 from the MetaPhlAn database is classified as *Phocaeicola* sp900546645 under GTDB taxonomy. There are no current publications referencing SGB1818, while the GTDB website shows three genomes all isolated from the human gut. Reclassifying to GTDB taxonomies adds a layer of interpretation to results.

Classification using MetaPhlAn and GTDB-tk showed a large number of unclassified reads. This is not surprising as other studies (Levin et al., 2021) have also shown a high proportion of unclassified reads. However, we wanted to be sure that there wasn't contamination, for example, with host prey. To identify eukaryotic reads were classified using Kraken2 by alignment to the large *nt* database containing genomes from bacteria, protozoa, fungi, plants and mammals (Wood et al., 2019). The parameters used were a *k*-mer length of 31 and read length of 130. Bracken was used on the Kraken2 output to estimate relative abundance of taxa while taking into account read length and reference genome size (Lu et al., 2017).

Statistical analyses were performed using R v4.1.2. Taxonomic alpha and beta diversity were calculated with vegan v2.6-4. Linear regression for differential abundance of taxa, principal coordinate analyses were carried out using the R base package. PERMANOVA was used to test for significant differences in MDS and t-tests for differences in alpha diversity (Li et al., 2022). Plots were generated in R using ggplot2 v3.4.4 and tidyverse v2.0.0. Phylogenetic trees were visualised with iTOL v655.. Beta diversity was quantified and visualised using Bray-Curtis dissimilarity index. Bray-Curtis dissimilarity was first quantified using MetaPhlAn classification, however due to the large abundance of unclassified reads, a reference agnostic approach was considered to confirm the results for beta diversity measurements. k-mer analysis was performed using Sourmash (Moore et al., 2022) on the pre-processed reads as a reference agnostic approach. Bray-Curtis dissimilarity index was also used on the k-mer results to quantify and visualise beta diversity. A linear regression model was used to determine the relationship between bacterial genera and the sex of the lion or season the sample was collected.

2.5.3 Assembly of MAGs

To classify potentially new taxonomies, pre-processed reads were assembled into MAGs using MEGAHIT (Li et al., 2016) and quality control was performed using Quast (Gurevich et al., 2013).

MEGAHIT was chosen over MetaSPades because MEGAHIT is specialised for shotgun sequence data while MetaSPades, which can assemble shotgun sequences, is more adapted to hybrid assembly with long-reads. Contigs with a completeness of 50% and above and contamination below 10% were retained for further analysis. See supplementary figure S4 for summary of MAG completeness and contamination. Contigs were then binned into groups based on taxonomy and similarity using MetaBAT, MaxBin and Concoct and then combined using DAStool (Sieber et al., 2018). Using all three binning tools was complementary, correcting potential errors or omissions caused by any of the three tools. The following parameters were used:

- MaxBin – contig length≥1 000, marker gene sets=107, probability threshold=0.9;
- MetaBAT – contig length≥2 500, ≥95% contig quality for each bin, bin size≥200 000; and
- Concoct – k-mer length 4, contig length≥1 000.

Further taxonomic classification was then performed by comparing binned MAGs to the Genome Taxonomy Database (GTDB) (Parks et al., 2022) using GTDK-tk (Chaumeil et al., 2022). GTDB classification was chosen so that read classification could be easily and accurately compared with MAG classifications. he novelty of MAGs was assessed by comparison against 5 596 genomes generated from 180 wild animal species (Youngblut et al., 2020) using dRep (Olm et al., 2017).

Lastly, functional analysis of the MAGs was performed using Prokka (Seemann, 2014) and GhostKOALA (Kanehisa et al., 2016). The HMM algorithm of Prokka (which uses Prodigal, discussed previously) is more accurate in protein inference than that of MetaGeneMark 2 Prokka has a stream-lined, mostly automated workflow. Although MetaGeneMark 2 is more specialised, the aims of this project were to assess the general functional composition of the lion gut microbiome rather than ask specific questions, although this data is still available for further analysis in the future. GHOSTKoala was preferred over KEGG Mapper and the COG database as its web-based design was much easier to use and saved time while its output was high quality and included pie charts of pathway abundance and bacterial and viral classification.

2.5.4 Classification of reads using MAG alignment

To supplement the classification of microbiome reads, pre-processed reads were compared to the created MAGs using the CoverM compare argument (Chklovski et al., 2023) to assess overall

coverage of the MAGs. CoverM provides a percentage coverage of each genome rather than classifying each read individually based on nucleotide similarity. For example, if five reads from a total of ten aligned to a genome, and said reads covered 10% of said genome, CoverM would report a value of 10% and not 50%. This approach particularly complemented the marker-gene based approaches used by MetaPhlAn4 and GTDB-tk; aligning reads to MAGs includes reads that may not align to specific marker genes, thereby providing a more comprehensive view of the microbial community. Checking for consistency between the percentage abundances obtained from the other classification tools and CoverM served as a cross-validation, increasing confidence in classifications. Moreover, this approach mitigated potential biases associated with marker gene selection.

3. Results

We were able to successfully extract DNA from all 23 samples. Additionally,data from three reference lions sampled in India from another study (Mittal et al., 2020) were re-analysed as comparator data hereafter referred to as reference lions. These lions were rescued from a zoo in India and returned to the wild before being darted for sampling.

3.1 Bacterial classifications and a core catalogue of the lion gut microbiome

3.1.1 MetaPhlAn

Reads sequenced from the lion gut microbiome samples were classified using three methods; MetaPhlAn, GTDB and MAG comparisons. First,MetaPhlAn 4 was used to classify the reads to the phylum and genus levels (Figure 4). The large abundance of unclassified reads at the phylum level (Figure 4A) is striking. Across the samples, an average 33.6% of the reads were unclassified, with samples ranging from 7.7% to 52.4% unclassified. Interestingly, all of the reads classified to the phylum level were also classified to the genus level (Figure 4B). In other words, the abundance of unclassified reads was the same for both the phylum and genus classifications. What was also interesting was that the reference data showed a similar percentage of unclassified reads (30.9%). The large proportion of unclassified reads did raise concerns about contamination in my data, possibly from prey DNA. However, the abundance of unclassified reads in the reference data supports the idea that this output was not specific to my data. To clarify if the unclassified reads were a factor of MetaPhlAn classifications, reads were aligned to the generated MAGs (see section 3.1.3).

Phylogenetic classification of the lion gut microbiome showed five phyla, in order of abundance: Bacteroidota (21.3%), Pseudomonadotaia (20.0%), Bacillota (13.7%), Fusobacteriota (7.5%) and Actinomycetota (3.9%) (Figure 4A). The three reference lions had the same five phyla present in their gut microbiome; however, Bacillota were nearly three times more abundant (30.2%), while Actinomycetota was similarly the least abundant (8.1%).

At the genus level the most abundant genera were two unclassified genus level genome bins (GGBS), GGB44117 (14.0%) and GGB1354 (14.2%) from the phyla Bacteroidota and Pseudomonadotaia, respectively (Figure 4B). The three reference lions had a much lower abundance for these genera (GGB44117 and GGB1354) of <1%. This was interesting as their phyla abundances had some similarities while their genus level classifications were more starkly contrasting.

Although the phyla of the GGBs was helpful for interpretations, the GGBs themselves provided little insight into why these genera might be so abundant in the lion gut microbiome. As a result of this new methodology for classifying reads implemented by the MetaPhlAn developers (Blanco-Míguez et al., 2023), there is little to no literature on the GGBs. For this reason the GGB taxonomy classifications were converted to Genome Taxonomy DataBase (GTDB) taxonomy, discussed in the next section (section 3.1.2).

Additionally, at the genus level, eight lions differed from the average with *Fusobacterium* (F9), *Cetobacterium* (F13), *Enterococcus* (F14), *Peptostreptococcus* (M1 & M3), *Bacteroides* (M4), GGB77017 (M6) and *Clostridium* (M8) as the most abundant genera. Of note, F9, F13, M1 and M8 were distinct outliers based on their abundances of above 20% for these genera. Notably, F9 and M8 were samples previously identified with lower DNA concentrations than recommended by the NICD. However, sample F2 was also sent at below recommended concentrations and followed a similar abundance profile to the other lion gut microbiome samples, indicating that these differences may not be caused by low DNA concentrations. Also of interest for these outliers was lion F9 who had recently given birth to cubs and lion F13 who was one of the oldest lions sampled. Pregnancy and age could possibly have influenced the gut microbiome.

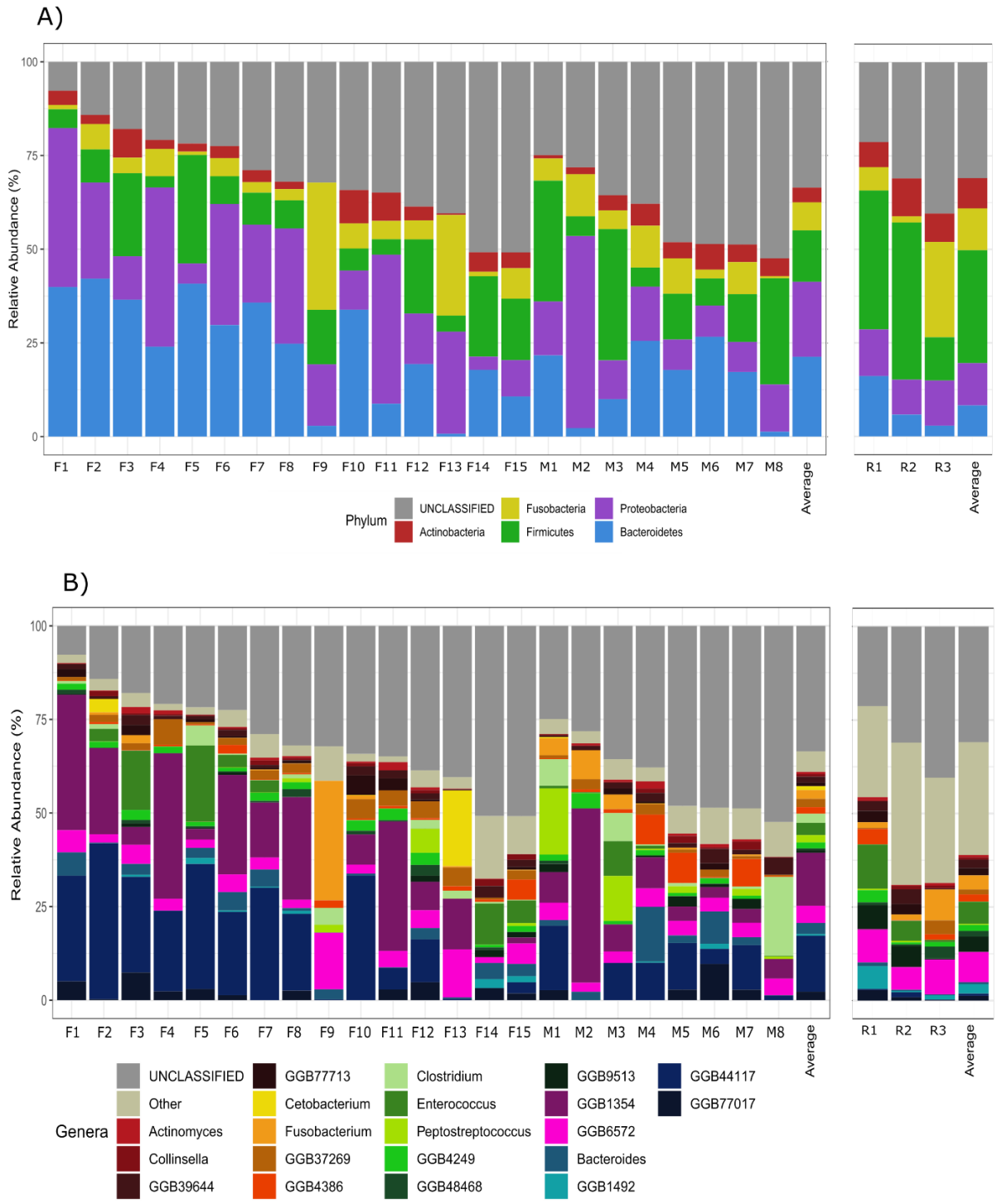

Figure 4: The lion gut microbiome is diverse and largely unclassified. A) Bar plot showing the relative abundance of bacterial phyla present in the *Panthera leo* gut microbiome from MetaPhlAn classification. **B)** Bar plot showing the relative abundance of the top 20 bacterial genera present in the *Panthera leo* gut microbiome from MetaPhlAn classification. The left block shows free-living lions darted in Namibia for this study while the right block shows three reference lions from India (Mittal et al., 2020). Genera are coloured by phylum using the colours shown in panel A. F = Female; M = Male; R = Reference lion

3.1.2 Genome Taxonomy Database

Converting MetaPhlAn taxonomy classifications to GTDB taxonomy clarifies the large abundance of unclassified reads and to aid in interpretation of results. The GTDB database has freely accessible genomes for every species as well as the origin of the genomes, which helped with interpretation. From an ecological perspective the database could be used to find animals with similar abundances of bacteria that could be compared behaviourally and phenotypically to lions. There are some taxonomic differences between the two databases as they put some genera in different phyla. Thus, converting from MetaPhlAn SGB taxonomy to GTDB taxonomy does provide additional interpretation, but also changed the relative abundance of bacterial phyla in the lion gut microbiome, in order of abundance: Bacteroidota, (37.2% previously 21.3%), Bacillota (12.6% previously 13.7%), Fusobacteriota (7.8% previously 7.5%), Pseudomonadotaia (6.0% previously 20.0%), and Actinomycetota (3.7% previously 3.9%) (Figure 5A). The most notable change was the reduction in abundance of Pseudomonadotaia and increase in Bacteroidota. The reason for the change in abundance of these two phyla was that GGB1354 (a Pseudomonadotaia and previously one of the most abundant genera) is converted to a genus of the phylum Bacteroidota named *Phocaeicola* under GTDB taxonomy, thus explaining the increase in Bacteroidota and decrease in Pseudomonadotaia. Conversion to GTDB taxonomy also resulted in an increase in Bacteroidota abundance in the three reference lions (14.4% previously 8.3%) while Bacillota remained the most abundant (24.8% previously 30.2%). Notably, the proportion of unclassified reads remained the same as with MetaPhlAn classification.

At the genus level the most abundant genera were *Bacteroides* (16.9%) and *Phocaeicola* (16.6%) (Figure 5B). However, three lions did not have *Bacteroides* or *Phocaeicola* as the most abundant genera. In these exceptions the most abundant genera were *Fusobacterium_A* (F9), *Cetobacterium_A* (F13) and *Clostridium_P* (M8). As mentioned previously, lion F9 was recently pregnant and lion F13 was one of the oldest lions sampled. What remained was the question of the high abundance of unclassified reads.

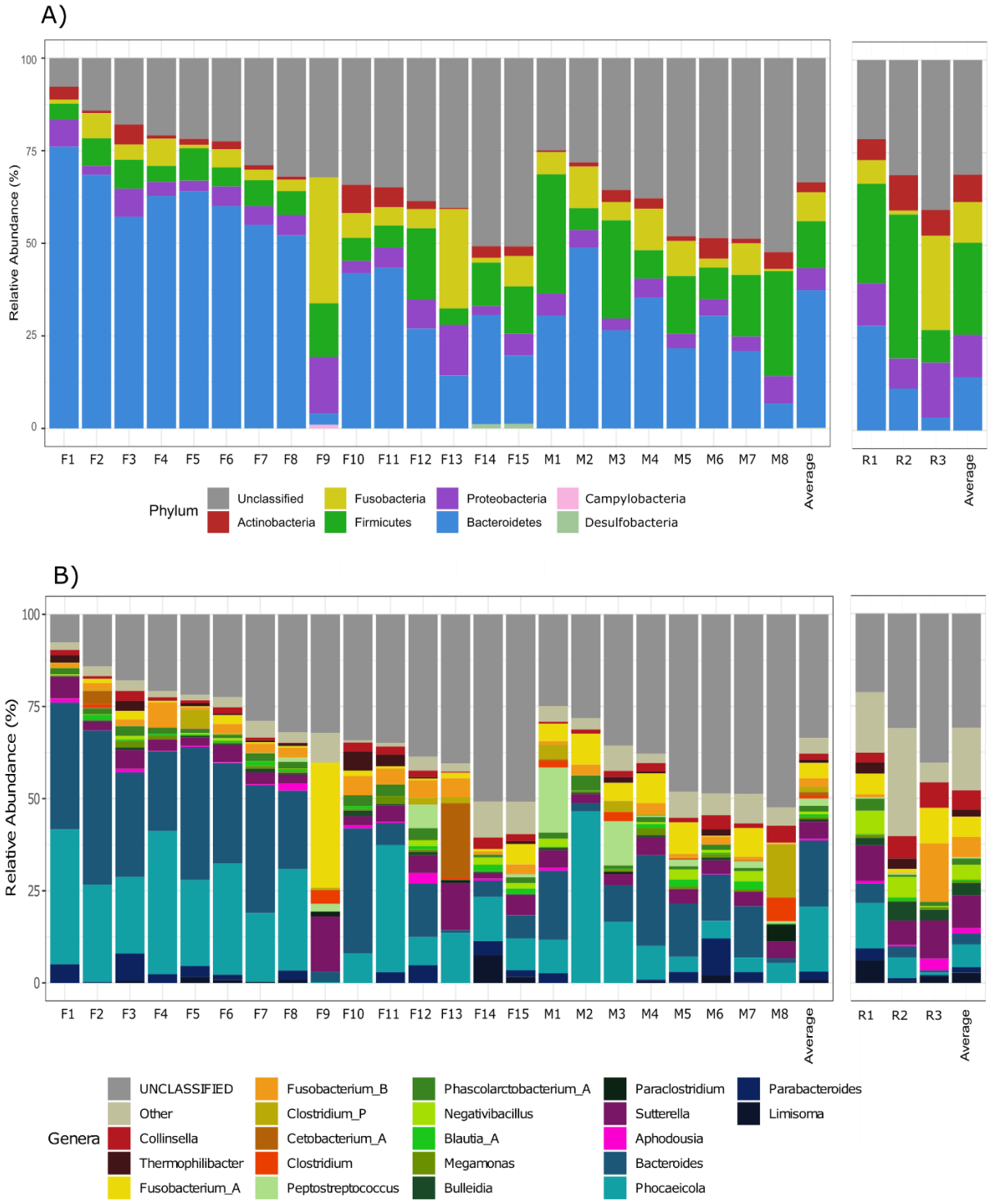

Figure 5: Conversions from SGB to GTDB database results in changes in taxon abundance at the phylum and genus level. A) Bar plot showing the relative abundance of bacterial phyla present in the *Panthera leo* gut microbiome from MetaPhlAn classification converted to GTDB taxonomy. **B)** Bar plot showing the relative abundance of the top 20 bacterial genera present in the *Panthera leo* gut microbiome from MetaPhlAn classification converted to GTDB taxonomy. The left block shows free-living lions darted in Namibia for this study while the right block shows three reference lions from India (Mittal et al., 2020). Genera are coloured by phylum using the colours

shown in panel A. F = female; M = Male; R = reference lion; SGB = species level genome bin; GTDB = genome taxonomy database

3.1.3 Kraken2 and Bracken to identify eukaryotic reads

Considering the fact that both MetaPhlAn classification and MAG alignment still showed over 25% unclassified reads, there was still the potential that these were caused by prey contamination. Therefore, reads were classified using Kraken2 and the *nt* (nucleotide) database; the largest Kraken2 database available (Table 2). This database includes genomes from bacteria, archaea, fungi, plants and animals.

Table 2: Kraken2 classification for reads sequenced from the gut microbiome of lions in Etosha, National Park.

Kraken2 (%)		Bracken (% of classified reads)	
Unclassified	41.4		
Classified	58.6	Bacteria	68.8
		Eukaryotes	27.1
		Viruses	4.0
		Archaea	0.1

In light of the proportion of unclassified reads (41.4%) observed in the Kraken2 analysis, it is evident that a considerable portion of reads in the lion gut microbiome remains unexplored or lacks representation in existing databases. Therefore, the unclassified reads must originate from previously undocumented species, highlighting the necessity for further exploration and expansion of reference databases to encompass a broader taxonomic diversity.

The abundance of eukaryotic reads (27.1% of classified reads) was notable. Of this 27.1% Animalia (24.4%) was the most abundant kingdom suggesting dietary elements in the lion gut persisting through DNA extraction. Arthropoda (17.6%) was the most abundant phylum within

Animalia and was mostly composed of class Insecta (17.3%), particularly from the order Lepidoptera (12.0%), implicating moths and butterflies. Lepidopterans may inadvertently be ingested by lions when they consume herbivorous prey species that feed on plants, where Lepidoptera larvae are commonly found (Ben-Ari and Inbar, 2013; Ray and Sunquist, 2001). Moreover, Lepidoptera may also be directly consumed by lions, especially during nocturnal foraging activities when these insects are active (Borstlap, 2002).

Additionally, within the mammalian sequences (2.4%) the order Carnivora (0.9%) and family Felidae (0.7%) were the most abundant. This likely indicates a small proportion of host contamination not removed indicating that the lions sampled had slight genetic differences from the reference lion genome taken from Ensembl.

3.2 Sex and season as causes of variability in the gut microbiome of lions

Using the MetaPhlAn species level data, Bray-Curtis dissimilarity index was measured to identify if the overall gut microbiome of lions differed between males and females. Bray-Curtis dissimilarity index measures differences in species composition between groups (Kers and Saccenti, 2022). MetaPhlAn classified data was chosen over the GTDB conversions since the MetaPhlAn database is more likely to discern smaller taxonomic distances between species. In other words GTDB may have a definition for one species while MetaPhlAn may split the category into two species. Although MetaPhlAn output is hard to analyse in the context of other papers, at a statistical level, it is more likely to identify differences between groups.

Using Bray-Curtis dissimilarity index, there was no significant difference in gut microbiome composition between male and female lions (P = 0.14; d.f. = 22)(Figure 6A). Due to the large proportion of unclassified reads, there was concern that this result may not be a true reflection of reality as there may be unidentified species causing significant differences between male and female lions. Therefore, a reference agnostic approach was used to confirm the findings. Sourmash was used to divide reads into k-mers and calculate Bray-Curtis distances between samples. The results confirmed no difference in gut microbiome composition between sexes (Figure 6B). However a change was seen in the clustering, indicating that the unidentified species would have an influence on the differences between sexes.

Shannon diversity was also measured. The Shannon diversity of the samples ranged from 1.74 to 2.4. There was no significant difference in Shannon diversity between the two sexes (P= 0.68; d.f. = 22) (Figure 6D). At a more specific level, two bacterial genera were different between males and females (Figure 6C). *Clostridium_AH* was more abundant in males (P = 0.007; d.f. = 22) while *Aphodousia* (P = 0.003; d.f. = 22) was more abundant in females.

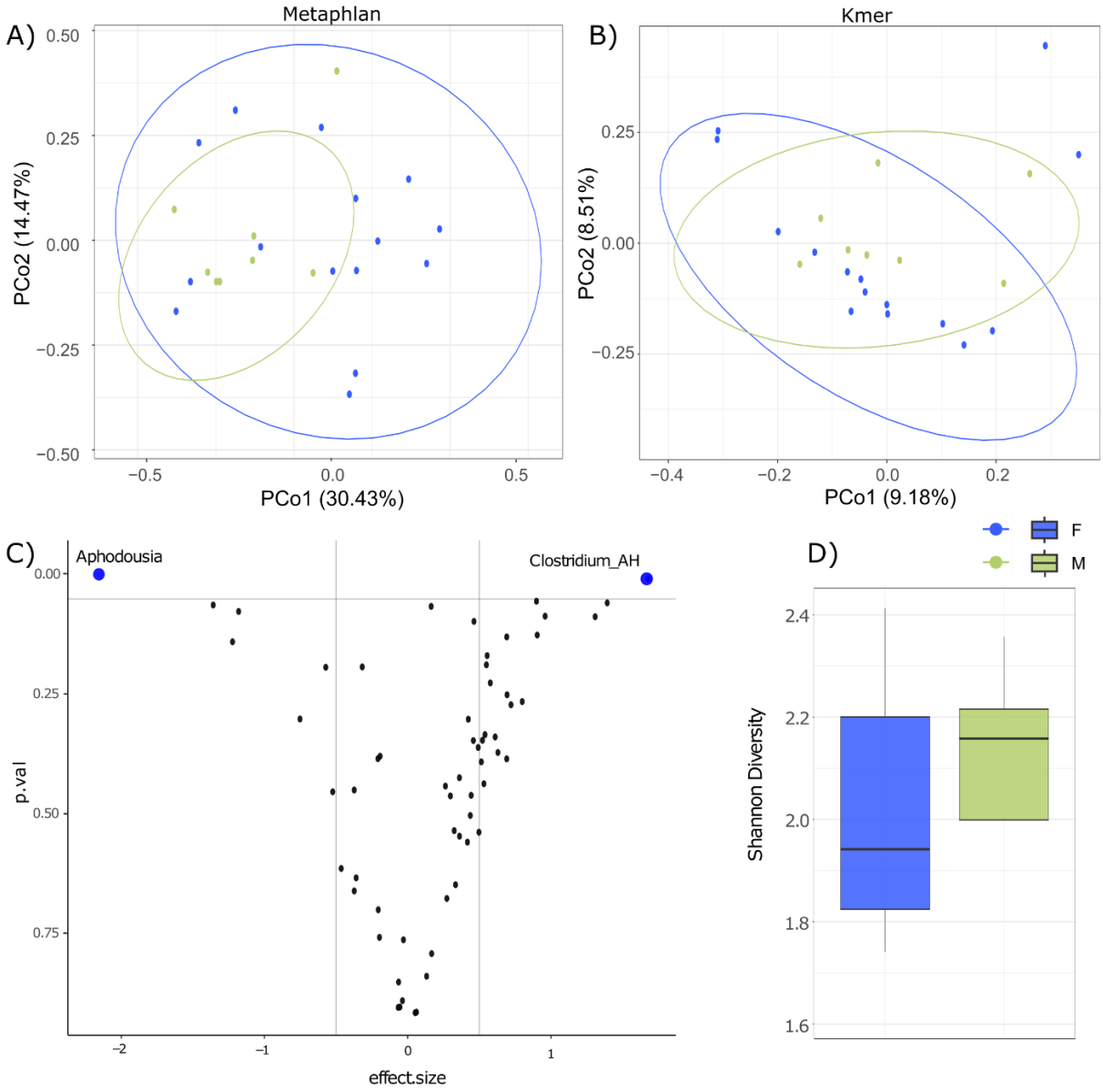

Figure 6: Sex explained little variance in the lion gut microbiome. Principal coordinates analysis (PCoA) plots the gut microbiome of male and female lions using **A)** weighted UniFrac distances of gut bacteria and **B)** k-mer distances. There was no significant difference between sexes. **C)** Volcano plot showing differentially expressed bacterial species in male vs female lions. The horizontal line represents a *p*-value of less than 0.05. Positive effect size indicates higher expression in males and negative effect size indicates increased expression in females. **D)** Shannon diversity of the gut microbiome of male and female lions using species counts.

Similar analyses were performed to identify differences between lions caught in winter and summer. There was no significant difference in the lion gut microbiome between lions captured in winter or summer (P = 0.63; d.f. = 22)(Figure 7A) using Bray Curtis dissimilarity . Once again, a reference agnostic approach confirms this finding. K-mer analysis similarly showed no significant difference between seasons (Figure 7B). There was also no significant difference in Shannon diversity between lions captured in either season (P = 0.18; d.f. = 22)(Figure 7D).

Some taxa were more abundant in lions captured in winter compared to those captured in summer and vice versa (Figure 7C). Lions captured in winter had a high abundance of *Plesiomonas* relative to those captured in summer (P = 0.008). All of the reads classified to the genus *Plesiomonas* were also classified to the species *P. shigelloides*. Lions captured in summer on the other hand had a high abundance of *Dysosmobacter* (P = 0.038; d.f. = 22), *Pelethomona*s (P = 0.021; d.f. = 22), *Metalachnospira* (P = 0.033; d.f. = 22) and *Clostridium* Q (P = 0.012; d.f. = 22) compared to those captured in winter. The only *Dysosmobacter* species identified was *D. welbionis* but the majority of reads were unclassified at the species level. No *Pelethomonas*, *Metalachnospira* or *Clostridium* Q species were identified.

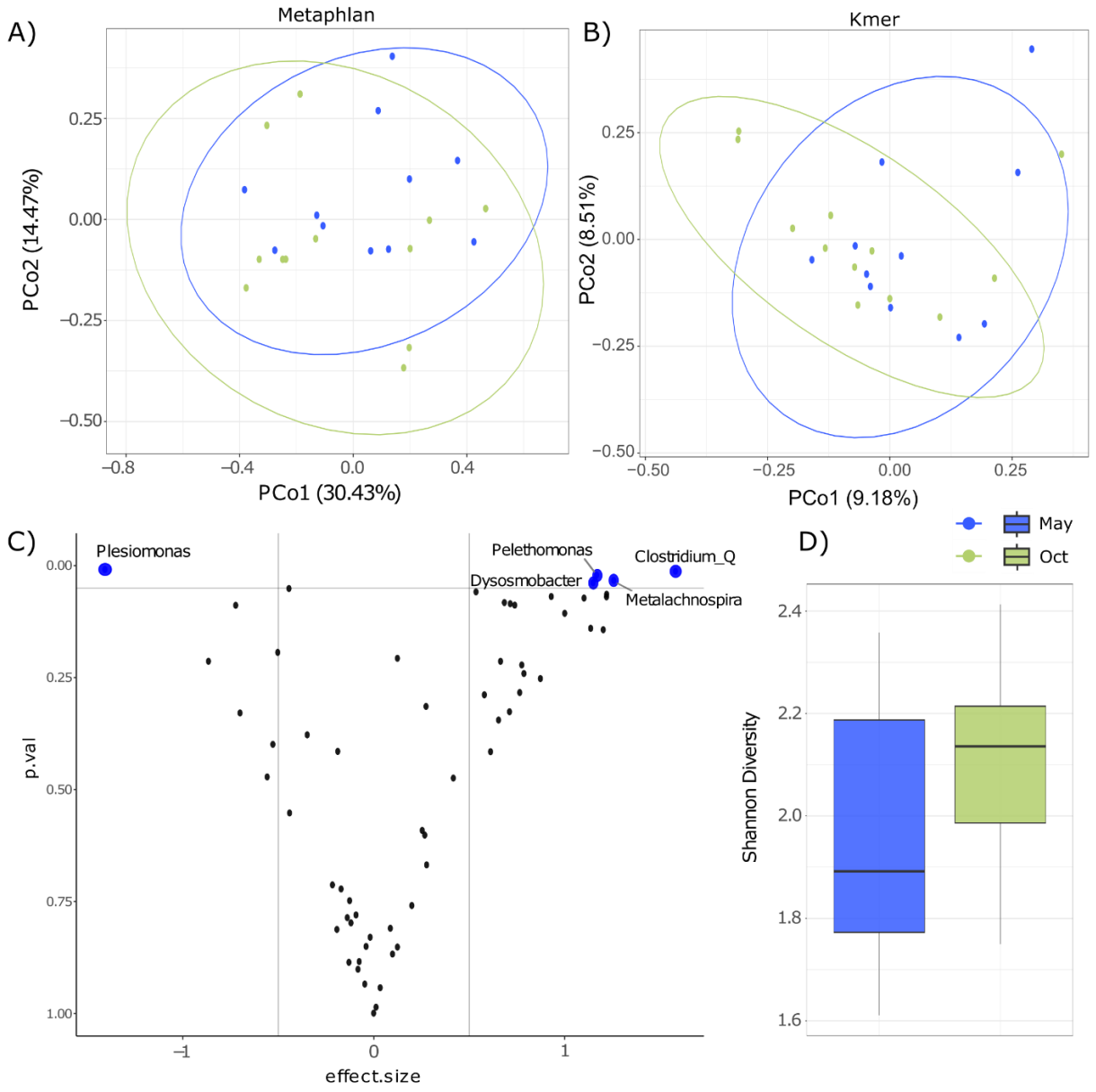

Figure 7: Season explained little variance in the lion gut microbiome. Principal coordinates analysis (PCoA) plots of lion gut microbiome composition from lion faecal samples collected in summer and winter using **A)** weighted UniFrac distances of gut bacteria and **B)** k-mer distances. There was no significant difference between sexes. **C)** Volcano plot showing differentially expressed bacterial species in lion gut microbiome samples collected in May (winter) vs October (summer). The horizontal lion represents a p-value of less than 0.05. Positive effect size indicates higher expression in summer and negative effect size indicates increased expression in winter. **D)** Shannon diversity of the lion gut microbiome in summer and winter using species counts.

3.3 Novel meta-genome assembled genomes

3.3.1 Assembly of novel meta-genome assembled genomes

Considering the large abundance of unclassified reads, and with no evidence of contamination, we hypothesise that the majority of the lion gut microbiome is composed of unclassified bacteria. To assess a possible contribution of new genomes to current databases, we assembled reads into Meta-genome Assembled Genomes or MAGs. Each MAG should represent the entire genome of a bacteria or other microorganism. Assembly of reads created 272 MAGs with a completeness of 50% and above and contamination below 10% (Figure 8). All MAGs were successfully classified using GTDB-tk to the phylum level, 268 to the genus level and 63 to the species level (Figure 8D). The low number of MAGs classified to the species level possibly could indicate that they originate from currently unclassified species.

To support the idea of novel genomes, we compared the lion microbiome MAGs to 5 596 reference genomes created from the gut microbiome of 180 wild animals (Youngblut et al., 2020). Of the 272 MAGs, 211 had less than 95% average nucleotide identity (ANI) with the reference MAGs, indicating that they are possibly new contributions to research, representing genomes from microorganisms that have never before been sequenced or classified.

At the phylum level, 94 MAGs were of the phylum Bacillota, 60 Bacteroidota, 46 Fusobacteriota, 38 Actinomycetes, 26 Pseudomonadotaia, 6 Campylobacterotaeria and 2 Desulfobacteria (Figure 8A). Interestingly, none of the MAGs in the phylum Actinomycetota were classified to the species level, which could indicate one or several novel species in the phylum. This is supported by the short phylogenetic distances between MAGs in this phylum, again indicating that they are all closely related and likely represent MAGs created from one or two unclassified species present in the lion gut. A similar pattern of closely related MAGs is present in the Bacillota C phylum, however these are all classified as the same species. The similar phylogenetic distribution in the Actinomycetota clade supports the idea that the majority of MAGs in the phylum Actinomycetota represent a single new species.

At the genus level, the highest number of MAGs were classified as *Collinsella* and *Fusobacterium* B, 29 for each (Figure 8C). *Collinsella* are Actinomycetota and so if the closely related MAGs in this phylum do represent a new species, it is likely a species within this genus.

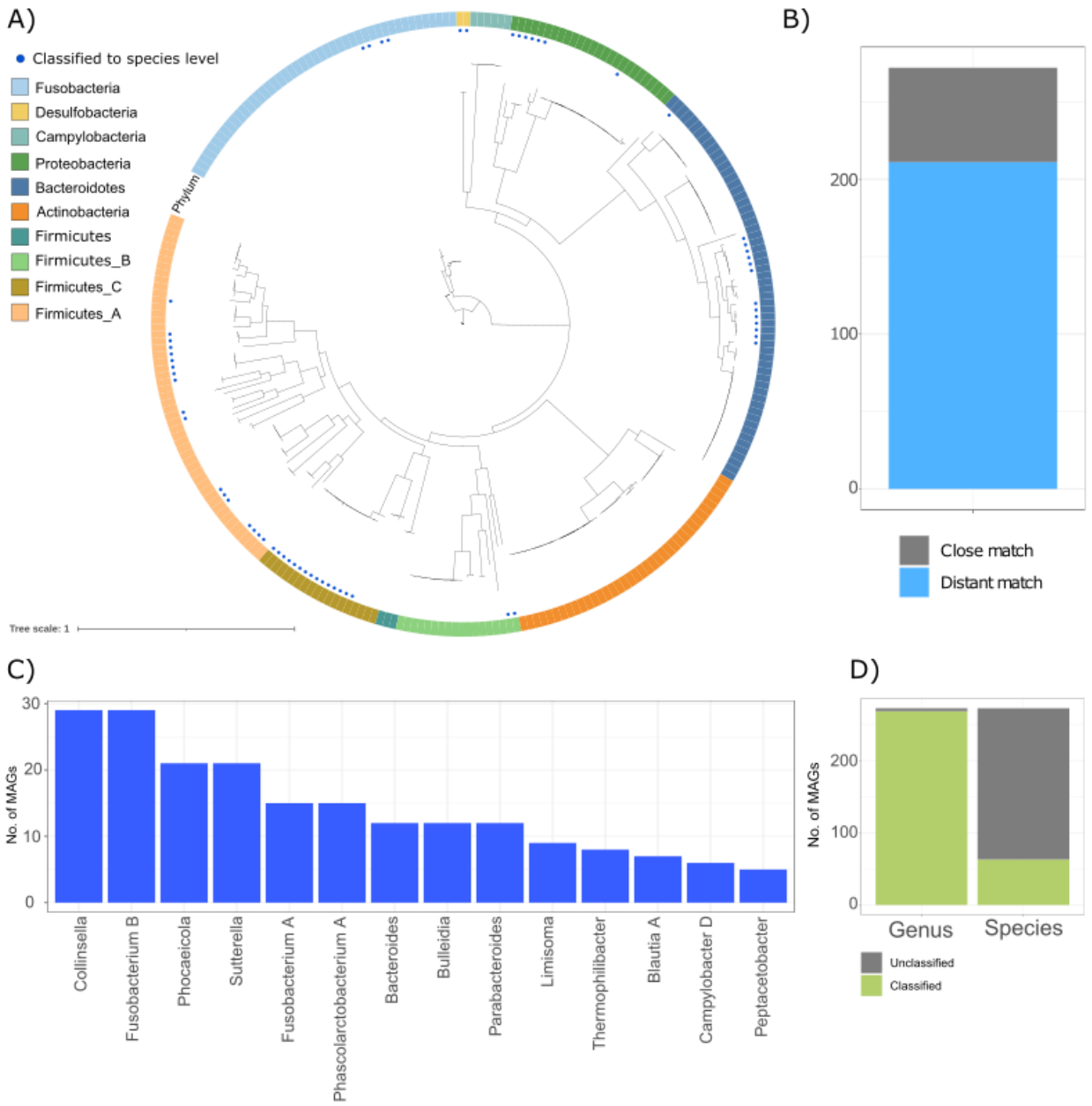

Figure 8: 272 metagenome assemble genomes (MAGs) were created from the lion gut microbiome. A) Phylogenetic tree showing the diversity of MAGs created from the lion gut microbiome. **B)** Clustering dendrogram showing MAGs with a close match (>95% average nucleotide identity) and a distant match (<95% average nucleotide identity) to 5 596 MAGs from a wild animal reference database **C)** Genus classification of MAGs. Genera assigned to 4 or more MAGs are shown. **D)** Number of classified and unclassified MAGs at the genus and species level.

3.3.2 Metagenome-assembled genomes alignment

To create another comparison for the MetaPhlAn and GTDB classifications, reads generated from shotgun metagenomic sequencing were aligned to the MAGs created above. MAGs were classified using GTDB to help with comparisons to the read classification data. As MetaPhlAn uses marker genes to estimate abundance, unclassified reads can still belong to microorganisms i.e. some reads may not be marker genes but still originate from a microorganism). All of the MAGs created in this study were classified as the kingdom Bacteria so if a large proportion of the reads were not marker genes but aligned to the MAGS, this would indicate that they still originated from microorganisms.We uncovered around 97% alignment of reads with *all* MAGs; however after filtering the MAGs for completeness of 50% and above and contamination below 10%, the proportion of unclassified reads was similar to MetaPhlAn and GTDB classification (34.8% previously 33.6%), which meant that the unclassified reads could still be caused by contamination. Hence, we decided to use a classification tool, Kraken2, with the large *nt* database containing sequences from bacteria, protozoa, fungi, plants and mammals (see section 3.1.4).

Classifying reads using comparison to classified MAGs also changed the relative abundance of bacterial phyla in the lion gut microbiome (Figure 9A). Percentage abundance is reported in brackets with MAG comparison first, then GTDB classification and finally MetaPhlAn classification. In order of abundance the identified phyla were; Bacteroidota (Percentage abundance is reported in brackets with MAG comparison first, then GTDB classification and finally MetaPhlAn classification.), (22.1% previously 37.2% for GTDB and 21.3% for MetaPhlAn), Actinomycetota (14.6% previously 3.7% and 3.9%), Bacillota (12.1% previously 12.6% and 13.7%), Fusobacteriota (10.8% previously 7.8% and 7.5%) and Pseudomonadotaia (4.5% previously 6.0% and 20.0%)(Figure 6A). The most notable change was the increase in abundance of Actinomycetota. As with the changes between MetaPhlAn and GTDB, the increase in this phylum was caused by an increase in one genus within the Actinomycetota phylum, namely *Collinsella*. At the genus level the most abundant genera were *Collinsella* (13.5%) and *Phocaeicola* (12.5%) (Figure 9B). The high abundance of *Collinsella* could be caused by an unbalanced proportion of MAGs, as the most common genus classification within the MAGs was *Collinsella*. Despite not answering the question of contamination, the similar abundances of genera other than *Collinsella* supports the reliability of the previous classification results.

As before, three lions were outliers as they did not have *Collinsella* or *Phocaeicola* as the most abundant genera. These exceptions were the same as before; the most abundant genera were *Fusobacterium_A* (F9), *Cetobacterium_A* (F13), and *Clostridium_P* (M8).

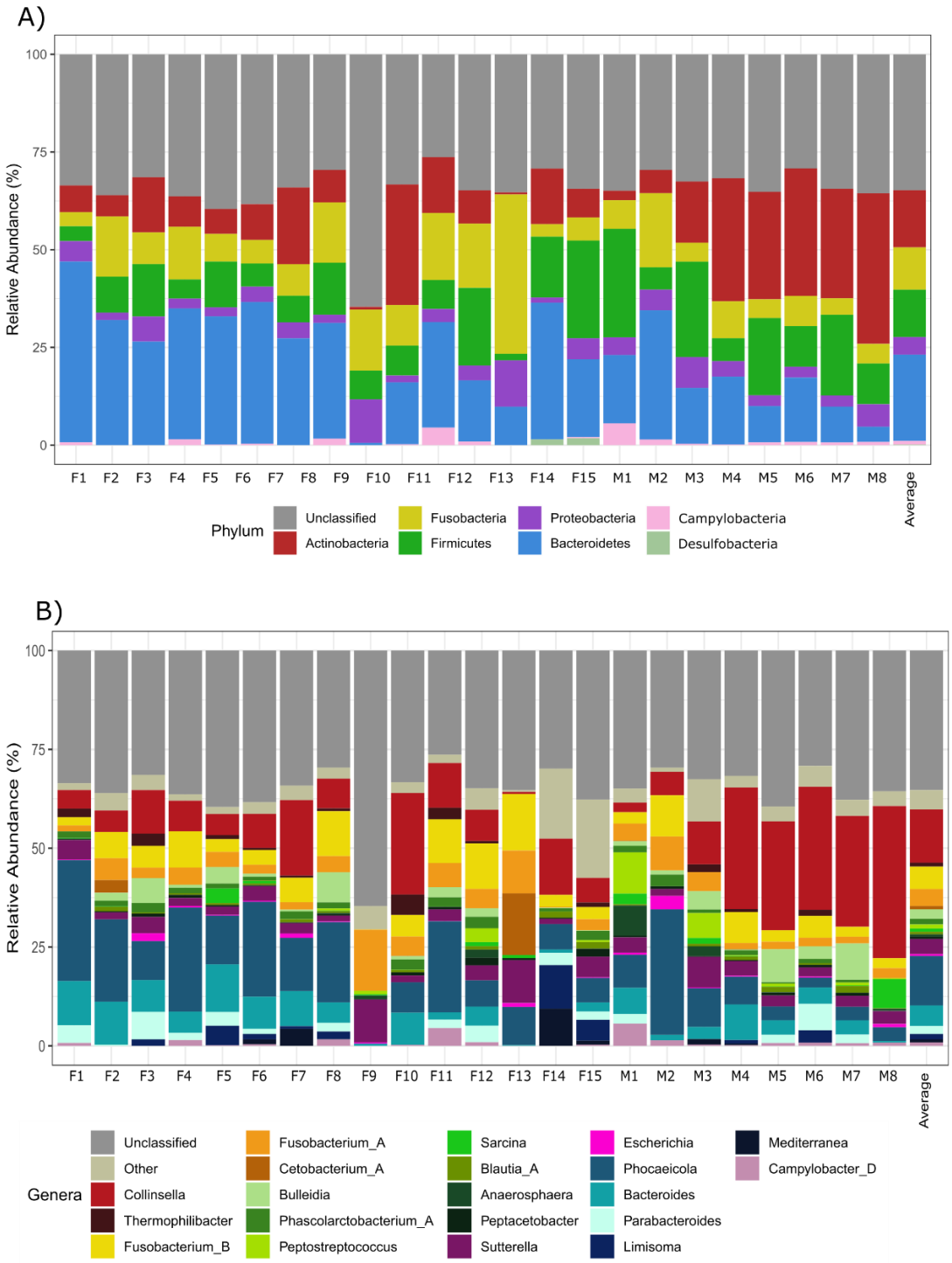

Figure 9: Classifying reads through metagenome assembled genomes (MAGs) may aid interpretations of results. A) Relative abundance of bacterial phyla based on comparison of reads obtained from the *Panthera leo* gut microbiome to MAGs created from the lion gut microbiome and classified using GTDB-Tk. **B)** Relative abundance of bacterial phyla based on comparison of reads obtained from the *Panthera leo* gut microbiome to MAGs created from the lion gut microbiome and classified using GTDB-Tk. Genera are coloured by phylum using the colours shown in A. F = female; M = Male; R = reference lion; GTDB = genome taxonomy database

3.4 Metabolism is a large component of the lion gut microbiome

To better understand the functionality of microorganisms in the lion gut, 27,916 reads were annotated using GHOSTkoala. We found 44.6% of reads were annotated to KEGG Orthologs (KO) and Pathways (Figure 10). Of the successfully annotated genes, nearly 80% of those found in the lion gut microbiome are related to metabolism (Figure 10A). Within the genes associated with metabolism, the most abundant pathway was global and overview maps (2 674 genes), in other words these genes are related to broad cellular functions, rather than being specific to a single, well-defined pathway. Carbohydrate (676 genes), amino acid (479 genes) and energy (262 genes) metabolism were the second, third and fourth most abundant pathways, respectively. We had originally hypothesised that the most abundant pathway in the lion gut microbiome would be amino acid metabolism considering that proteins compose the majority of their diet. However, the most abundant pathway being carbohydrate metabolism is still logical as carbohydrates from prey tissue and some plant carbohydrates do form a large complement of the lion diet (Borstlap, 2002). Outside of metabolism, environmental information processing (555 genes), genetic information processing (252 genes), and cellular processes (266 genes) had the highest abundances of associated genes.

Considering the high purine diet of lions, nucleotide metabolism (150 identified genes) was investigated more specifically. Previous research has shown that meat-eating carnivores have a higher percentage of genes involved in purine metabolism compared to herbivores (Zhu et al., 2018). Under nucleotide metabolism, purine metabolism was analysed through guanine, adenine and urate degradation pathways (Figure 10B). Metabolism of guanine monophosphate (GMP) and adenine monophosphate (AMP) could be entirely performed by the lion gut microbiome except for the conversion of urate to 5-hydroxyisourate. In this case, both urate oxidase and flavin adenine dinucleotide (FAD)-dependant urate hydroxylase were missing from KEGG annotation. Wehypothesise that this step is facilitated entirely by the lion's metabolism. In some cases one reaction was missing from the lion gut microbiome but alternative paths in purine and urate metabolism were present. For example, conversion of allantoate to urea would not have been possible using identified genes but conversion to ureidoglycine, euridoglycolate then urea would have been possible.

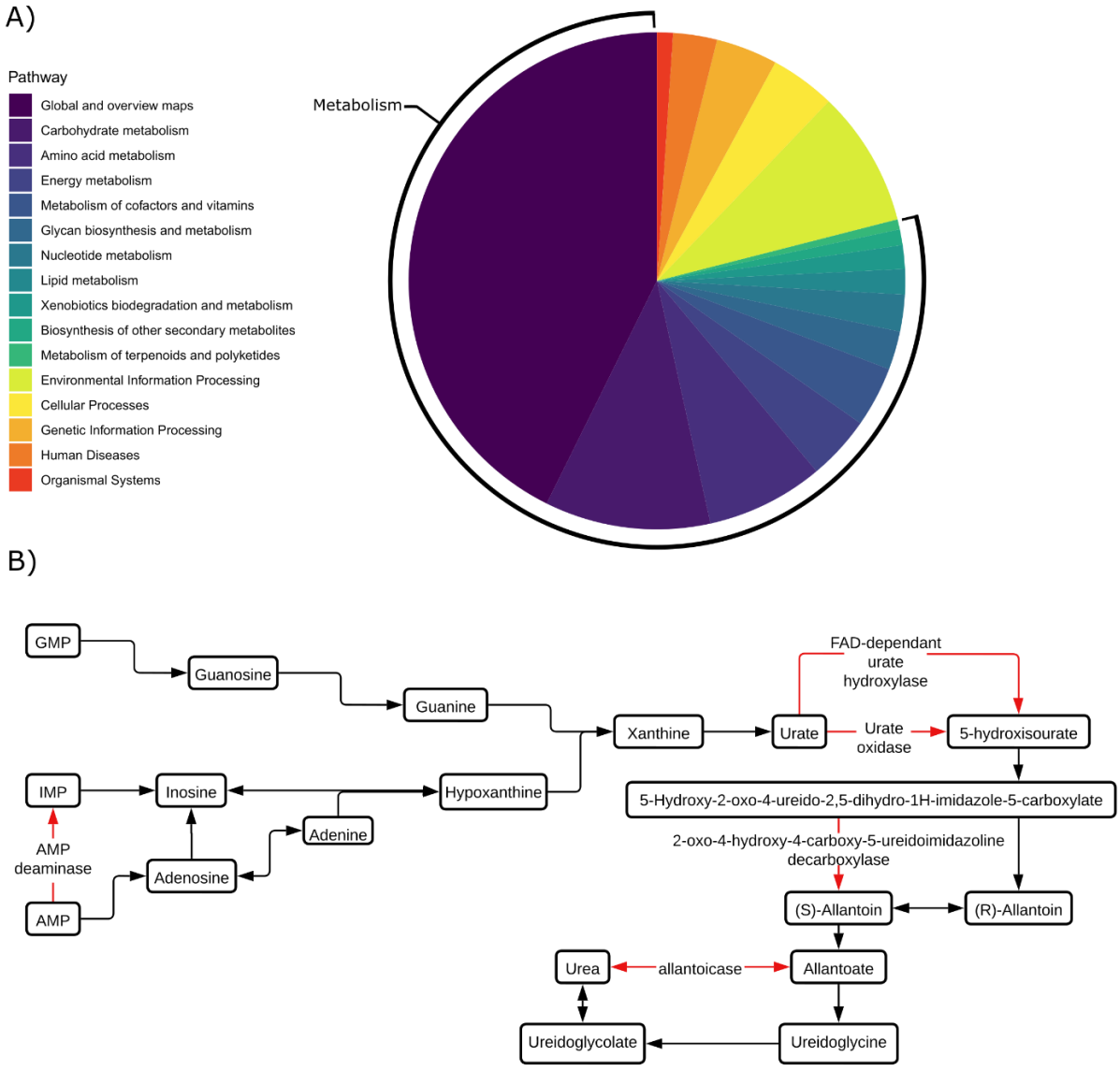

Figure 10: The lion gut microbiome has a large proportion of genes involved in amino acid and purine metabolism. A) Pie chart showing the distribution of KEGG pathways present in the gut microbiome of lions. Counts are based on number of proteins per pathway. **B)** Overview of purine metabolism and urate degradation pathway. Red arrows indicate a gene not found in the 23 lion gut microbiomes.

4. Discussion

4.1. Characterising the lion gut microbiome

4.1.1 Core taxa

On average, the most abundant genus in the lion gut was *Bacteroides* (GGB44117 under MetaPhlAn taxonomy), which is in contrast to the reference lions sampled in India where *Fusobacterium* was the genus with the highest abundance (Mittal et al., 2020). When analysed through the Nextflow pipeline used in this study, *Sutterella* and *Phocaeicola* were the most abundant genera for the reference lions.

There are multiple possible reasons for the difference in bacterial gut genera between Asiatic lions (*Panthera leo leo*) and African lions (*Panthera leo melanochaita*). Firstly, the gut microbiome is known to differ between hosts from different geographies, for example humans (Maghini et al., 2024), mice (Goertz et al., 2019) and some carnivores (Adams et al., 2021; Colborn et al., 2020). The differences between these lions could be due to their different environments. Secondly, the lions in this study were born in the wild and have been free-roaming their entire lives, while the reference lions were captured and spent a part of their lives in a circus before being released (Youngblut et al., 2019). This difference in background is another possible cause for differences in the gut microbiome of these host subspecies. Finally, it is possible that changes in bacterial genera in the gut were caused by their allopatric separation over a prolonged period of evolutionary history (de Manuel et al., 2020). It would make sense for lions globally to have a similar proportion of high level taxa such as phylum due to their shared ancestry but differ at lower levels such as genus caused by phylogeographic changes over time. Current data supports the idea that physical distance prevents bacterial dispersal between species which in turn accelerates compositional divergence between the gut microbiomes of host species over time (Moeller et al., 2017; Nishida and Ochman, 2018). Allopatric separation is a plausible explanation for the differences in abundance between the lions native to Namibia and India as they still retain some similar abundances of bacteria common to both subspecies. For example, both groups have a high abundance of *Phocaiecola*, which may be a conserved genus within the gut microbiome of lions for functional reasons, thus it is retained in similar abundance regardless of geography because it

is necessary for the survival of the species. On the other hand, genera such as *Bacteroides*, *Sutterella* and *Fusobacterium* were able to shift over time either because they perform similar functions within the lion gut or because they do not serve an essential function within the lion gut. Indeed, temporal changes in the carnivore gut microbiome are well-documented, with studies indicating substantial fluctuations in *Bacteroides* abundance over years (Rojas et al., 2022).

In the current study, *Bacteroides* was the most abundant genus in the gut microbiome of free-living lions in Etosha National Park. *Bacteroides* is often the most abundant genus in the mammalian gastrointestinal tract and, in some studies, is the only genus common between herbivores, carnivores and omnivores (Wu et al., 2022). *Bacteroides* species are Gram-negative, obligate anaerobic, non endospore-forming bacilli (Karlsson et al., 2011). They can be motile or non-motile. *Bacteroides* species are involved in various metabolic activities, including the degradation of complex carbohydrates and short-chain fatty acid (SCFA) production (Houtman et al., 2022). Indeed, humans with a high intake of protein and animal fat in their diet have a high abundance of *Bacteroides* (Wu et al., 2011). Similarly, a number of carnivores with high protein and fat diets have high abundances of *Bacteroides* (percentage abundances are mentioned in brackets), including black-backed jackals (*Canis mesomelas*) (15.1% and 15.76%) (Menke et al., 2014; Wu et al., 2022), wild wolves (*Canis lupus*) (~16%) (Zhang and Chen, 2010), dholes (*Cuon alpinus*) (13.58%) (Wu et al., 2022) and raccoon dogs (*Nyctereutes procyonoides*) (36.91%) (Wu et al., 2022). Excluding raccoon dogs, which had a particularly high abundance of *Bacteroides*, the other species have a strikingly similar percentage abundance to the 23 lions in this study, of between 13 and 16%.

However, *Bacteroides* is not the most abundant genus in the gut of all carnivores. Cheetahs (*Acinonyx jubatus*) have *Clostridium* (24.5% and 19.5%) (Menke et al., 2014; Wasimuddin et al., 2017) in highest abundance in their gut and a comparatively low abundance of *Bacteroides* (5.4%) (Menke et al., 2014). Similar findings were true for spotted hyena (*Crocuta crocuta*), where *Clostridium* (17.88%) was most abundant and *Bacteroides* (3.38%) less so (Rojas et al., 2022).

A notable distinction between lions and cheetahs is their social vs solitary natures; lions exhibit a cooperative, pride-based hunting strategy, whereas cheetahs typically hunt and consume prey individually (Hilborn et al., 2018). Other carnivores such as jackals, wolves, and dholes, which share a similar abundance of *Bacteroides* to lions, also engage in pack hunting (Barber-Meyer et

al., 2016; Hayward et al., 2017; Khatiwada et al., 1970). This difference in social organisation could have a variety of effects on the gut microbiome. Firstly, pack-hunting animals are more likely to horizontally transfer bacterial species between individuals, especially in animals with a fluctuating pride structure, like lions (Sarkar et al., 2020). In contrast, solitary hunters have limited interactions with conspecifics, resulting in lower rates of bacterial exchange (Logan et al., 2010). Secondly, pack hunting vs solitary hunting affects dietary patterns: solitary hunters consume the most nutritious portions of prey immediately, while pack hunters usually distribute resources based on social hierarchies (Hayward and Kerley, 2008). Finally, these different hunting tactics have different dietary requirements, for instance, cheetahs' reliance on short bursts of energy necessitates a high protein intake, whereas lions prioritise a diet rich in both protein and fat (Hayward and Kerley, 2008). These differences all affect the gut microbiome and together may be the reason for a higher abundance of *Bacteroides* in the gut of pack-hunters compared to solitary hunters.

However, solitary hunting cannot be the only explanation for reduced abundance of *Bacteroides* in the carnivore gut as hyaena also have low *Bacteroides* abundance, despite their large fission-fusion clans (Smith et al., 2007). Dietary differences may be a more plausible explanation in the case of hyaena as they are scavengers and opportunistic predators (Watts and Holekamp, 2007). They consume bones and other parts of prey not typically eaten by other carnivores.

The majority of *Bacteroides* reads were classified as Bacteroides sp. 900766005 (13.8%). There are only two genomes for this species on the GTDB database, both of which derived from human gut samples collected in China (Nayfach et al., 2019). *Bacteroides stercoris* (2.63%), *B. fragilis* (0.22%) and *B. uniformis* (0.17%) were also identified species. These are all generally commensal bacteria which do not differ greatly from the metabolic activities of *Bacteroides* mentioned previously (López-Almela et al., 2021; Ryu et al., 2023), although *B. fragilis* has been shown to be pathogenic in some instances (Yekani et al., 2020).

The second most abundant genus in the lion gut microbiome was *Phocaeicola* (16.5%) (GGB1354 under MetaPhlAn taxonomy). *Phocaeicola* is in the same family as *Bacteroides*. In fact, recently 14 *Bacteroides* species *(B. vulgatus, B. barnesiae, B. caecicola, B. caecigallinarum, B. chincillae, B. coprocola, B. coprophilus, B. dorei, B. gallinaceum, B. massiliensis, B. paurosaccharolyticus, B. plebeius, B. salanitronis, B. sartorii*) have been reclassified as *Phocaeicola* due to phylogenetic

differences (García-López et al., 2019). New modifications describe the phenotype of this genus as Gram-negative, non-spore-forming, coccoid to rod-shaped cells with no flagella (García-López et al., 2019). Considering this recent transfer of *Bacteroides* species to the *Phocaeicola* genus as well as these two genera sharing the same family (*Bacteroidaceae*), *Phocaeicola* bacteria share a genetic lineage and functional genes with *Bacteroides*.

The majority of the *Phocaeicola* reads in this study's samples were not classified to the species level (16.3% of the total 16.5%). There are three possible reasons for this. Firstly, the lion gut microbiome could contain a diversity of *Phocaeicola* species which have inhabited the same environment, the lion gut, thus allowing for a significant amount of horizontal gene transfer over time. Transfer of genes would make each species hard to identify from another. Alternatively, the lion gut may be colonised by an unclassified species of *Phocaeicola* thus the MAGs from this genus would represent a new species that could supplement databases. The idea of a new *Phocaeicola* species is supported by the closely related Bacteroidota MAGs in the phylogenetic tree that are not classified to the species level. Thirdly, this species of *Phocaeicola* in the lion gut may be classified but mostly unknown and underrepresented in databases. In this case the MAGs classified in this study could contribute to strengthening the database and providing more reliable marker genes. It is also possible that a combination of these reasons may be true. For example, a significant amount of horizontal gene transfer could have led to a new species of *Phocaeicola* within the lion gut.

The high abundance of *Phocaeicola* in both the African and Asiatic lion gut microbiome is in contrast to previous research which found *Fusobacterium* to be the most abundant genera in the Asiatic lion gut microbiome (Mittal et al., 2020). One reason for *Fusobacterium* decreasing in abundance after reanalysis could be the changes in database structure since 2014 and 2024 (Lobanov et al., 2022). Changes in the abundance of genera are expected as different microbiome databases and tools can lead to differing results, with differences increasing overtime as databases start to include a wider range of sources (Sengupta et al., 2023; Smith et al., 2022). Although *Fusobacterium* was no longer the most abundant genus after reanalysis of the Asiatic lions, this genus was still present in the gut microbiome of almost all lions. *Fusobacterium* is a genus of obligate anaerobic, Gram-negative, non-sporeforming bacteria (Broadley and Schweon, 2017). Species from this genus have been found in high abundance in the carnivore gut microbiome (Zhu

et al., 2018), including domesticated dogs (Alessandri et al., 2019), meerkats (*Suricata suricatta*) and seals (family Otariidae) (Milani et al., 2020).

4.1.2 Unclassified reads

The high number of unclassified reads was initially of concern. Despite comparing the reads to large reference databases, on average, nearly 35% of the reads were unclassified. To confirm whether these unclassified reads were bacterial or potentially coming from the lions themselves or their prey, reads were compared against the most comprehensive database provided by Kraken2, the *nt* database. Only a small fraction of the unclassified reads were associated with mammalian DNA. The family Felidae was the most abundant of the mammalian reads, which likely indicates a small proportion of host reads not removed during pre-processing suggesting that the lions sampled had slight genetic differences from the reference lion genome taken from Ensembl. This is expected as reference genomes cannot account for all small mutations between individuals of the same species and will almost always have some differences between sampled individuals (Herrero et al., 2016).

The most interesting finding from the Kraken2 analysis was the large abundance of the phylum Arthropoda, suggesting dietary elements in the lion gut persisting through DNA extraction. The order Lepidoptera (composed of butterflies and moths) stood out for its particularly high abundance. These insects may be ingested by lions when they consume herbivorous prey species who are known to ingest insects themselves, either purposefully or incidentally (Ben-Ari and Inbar, 2013; Ray and Sunquist, 2001). Moreover, Lepidoptera may also be directly consumed by lions (Borstlap, 2002). Inclusion of non-microbial reads was a possibility since a shotgun sequencing approach was chosen. (Laudadio et al., 2018). It is common for host and dietary reads to appear post-sequencing using shotgun sequencing (Pereira-Marques et al., 2019; Rajar et al., 2022); however these are usually removed during pre-processing. The presence of Lepidoptera reads could have been an artefact of the pre-processing pipeline being designed to exclude lion reads. Considering that mammals share around 90% of their DNA (Crow, 2002), the removal of lion reads likely removes other mammalian reads, inadvertently allowing the retention of insect sequences, including those from Lepidoptera. Computational quality control assessments are known to have biases and are still on their way to perfection (Quince et al., 2017). Nonetheless the

abundance of Lepidoptera did not conflict with the bacterial classification results as the abundances of bacteria using Kraken2, MetaPhlAn and GTDB, remained similar.

Having shown a similar abundance of unclassified and bacterial reads in the lion gut microbiome samples, we are therefore confident that the majority of unclassified reads were from novel, previously unclassified microorganisms and that the bacterial reads were correctly classified.

4.1.3 Outlier taxa

Fusobacterium_A (33.9%) was the most abundant genus in the gut of lion F9. The majority species identified was *Fusobacterium_A* sp. 900015295 (31.9%). Five genomes of Fusobacterium_A sp. 900015295 are available on the GTDB database, four of which were collected from human faecal samples and one from an American black vulture (*Coragyps atratus)* (Roggenbuck et al., 2014). The lion with an abundance of Fusobacterium_A sp. 900015295 was sampled twice. The first time she was sampled (F10), she was pregnant and by the time she was sampled again five months later, she had given birth (F9). Post-pregnancy, the abundance of *Fusobacterium_A* in her gut increased more than ten-fold.

This female lion's unusual microbiome composition could be due to recently giving birth and the circumstances post-birth. Firstly, lionesses isolate from prides to give birth and remain with their cubs for about 6-8 weeks before rejoining a pride (Rudnai, 1973). Some lionesses may spend this period of isolation together and hunt together (Rudnai, 1973). This isolation from the pride could have affected her gut microbiome composition both due to her change in diet from hunting alone and sharing food with her cubs as well as no longer exchanging bacteria with other lions in her pride. Secondly, cubs suckle for around 6-15 months after birth (Rudnai, 1973), indicating that this lioness would still have had suckling cubs during the time the second sample was collected. Lioness milk contains fats, proteins and carbohydrates (De Waal et al., 2004). The production of milk could have affected this lionesses' metabolism and therefore her gut microbiome. This is supported by shifts in microbial communities between pregnant and lactating individuals of other species such as pigs (Liu et al., 2019) and primates (Hanning and Diaz-Sanchez, 2015; Sun et al., 2021). The increased abundance in *Fusobacterium* specifically is similar to what is seen in the pig (*Sus domesticus*) gut microbiome postnatally (Cheng et al., 2018; Sun et al., 2020). This genus is linked to gut inflammation in humans and pigs (Forbes et al., 2016).

Another outlier bacterial genus, *Cetobacterium_A* of the phylum Fusobacteriota was most abundant in the gut of lion F13 (20.23%). No reads from this genus were classified to the species level. *Cetobacterium* bacteria are short pleomorphic, non-spore-forming, rod-shaped cells which specialise in fermentation of peptides and carbohydrates with the end-product of acetate (Edwards et al., 2015). *Cetobacterium* species are associated with the gut microbiome of carnivorous fish (Medina-Félix et al., 2023), carnivorous birds (Boukerb et al., 2021), older otters (*Lutra lutra*) (Okamoto et al., 2021) and adult egyptian mongooses (*Herpestes ichneumon*) (Pereira et al., 2020). Lion F13 was 10 years old at the time of capture, making her the oldest of the lions. The high abundance of *Cetobacterium* could be attributed to her age considering that it is abundant in older individuals of other species (Okamoto et al., 2021; Pereira et al., 2020). However, the second oldest lion (male sample M2 and M7) did not show elevated Cetobacterium species, possibly highlighting age and sex differences. (Chen et al., 2020; Pafčo et al., 2019).

Alternatively, the high abundance of *Cetobacterium* in fish and fish-eating carnivores, might indicate that lion F13 may have a diet with some wild fish (Boukerb et al., 2021; Medina-Félix et al., 2023). Some lions are known to have a consistent fish component to their diet, although these are far more prevalent in lions with territory in coastal parts of Namibia than those found in Etosha National Park (Stander, 2019). Bracken analyses revealed that this lion had 2.3% of reads classed as fish (Actinopteri) in her gut compared to an average of 1.8% in the other lions.

In lion M8 *Clostridium perfringens* (14.3%) was the most abundant species. This species can be pathogenic with the presence of certain toxins: alpha, beta, epsilon and iota toxins and the beta-2 toxin and enterotoxin (Silva et al., 2014). Presence of *C. perfringens* and its toxins has been associated with diarrhoea in humans (Uzal et al., 2014), cats (Sabshin et al., 2012) and dogs (Silva et al., 2013). There is evidence that *Clostridium* toxins are metabolised in the carnivore gut (Levin et al., 2021). We were also not able to determine the presence or absence of toxins in the lion gut. Further research would be useful to assess whether Clostridium perfringens is associated with diarrhoea in carnivores.

In addition to the three lions mentioned as outliers, comparison of reads to classified MAGs caused a perceptible increase in *Collinsella* abundance, a genus of the phylum Actinomycetota. The fact that this genus was not found in as high abundance in the other two classification methods (MetaPhlAn and GTDB) is likely caused by the unbalanced abundance of MAGs. MetaPhlAn and

GTDB have more extensive databases compared to the 272 MAGs generated here. Additionally, *Collinsella* MAGs were the most common classification from the MAGs in this study. The abundance of *Collinsella from* MAG comparison is sensible considering similar carnivores; amur tigers (*Panthera tigris*) (He et al., 2018) and snow leopards (*Uncia uncia*) (Zhang et al., 2015) do have a high abundance of this genus.

4.2. Influence of sex and season on microbiome composition

I found no significant difference in overall classification, read composition or alpha diversity between sexes or seasons. Similar lack of differences between sexes in gut microbiome composition and alpha diversity have been found in cheetahs (*Acinonyx jubatus*) (Wasimuddin et al., 2017). While male and female lions differ in their hierarchical feeding habits, the feeding habits and pride compositions of lions is more complex than simple hierarchy; all-female prides are common, prides are often split into smaller groups, cooperative hunting is not always preferred (many solitary hunters exist) and sometimes pride hierarchies are not apparent (Rubenstein and Wrangham, 2014). The flexible nature of lion prides is likely to cause changes in diet and interactions between individuals, thus affecting ingestion of microorganisms and horizontal transfer of gut microorganisms respectively. Variable social structures could therefore reduce sex differences in microbiome diversity. Finer resolution analyses highlighted some differences in specific bacterial taxa between sexes and seasons.

4.2.1 Sex

Aphodousia was the only genus more abundant in female lions ($P = 0.003$) compared to male lions. No species was identified for *Aphodousia* and this genus has previously only been identified in the chicken gut microbiome (Gilroy et al., 2021); however in the GTDB database 12 of the 20 *Aphodousia* species belong to the genus *Sutterella* under the NCBI taxonomy. *Sutterella* has been found in low abundance in raccoon dogs (Kaneko et al., 2023), cheetahs (Menke et al., 2014) and black-backed jackals (Menke et al., 2014). Research on the human gut microbiome indicates this genus to be mostly commensal with moderate inflammatory consequences and a possible immunomodulatory effect (Hiippala et al., 2016). Pregnancy likely increases gut inflammation (Cheng et al., 2018; Sun et al., 2020).

Clostridium on the other hand was more abundant in male lions compared to females (P =0.007). This genus has been discussed previously in the context of *C. perfringens*, a possibly pathogenic species. However in this case the species *Clostridium fallax* was more abundant in males. This species has been identified as pathogenic in humans (McClane et al., 2012), sheep (Vatn et al., 2000), chickens (Cooper et al., 2013; Ellwood and Halliwell, 1973) and horses (Shaw et al., 2020), causing food poisoning and diarrhoea. Further investigation into pathogenicity of this species in male lions is warranted.

4.2.2 Season

Plesiomonas was more abundant in faecal samples collected from lions during winter compared to those samples collected in summer. All of the reads of this genus were classified as *P. shigelloides*, currently the only species in this genus. *P. shigelloides* is a pathogenic species in humans and other animals, causing gastrointestinal illnesses (Santos et al., 2015). This species is often transmitted through water sources. During the dry season when water is scarce, lions may be forced to drink from stagnant or unclean water sources, thus increasing the infection risk from *P. shigelloides*. Monitoring bacteria in water sources during the dry season could provide insight into possible sources of the pathogen and aid lion and ecosystem health.

Four genera had higher abundance in the wet summer season compared to the dry winter season: *Dysosmobacter*, *Clostridium_Q*, *Pelethomonas* (also classified as *Oscillibacter* under NCBI taxonomy) and *Metalachnospira*. These genera are related to metabolism and breakdown of fats (Roy et al., 2022), glucose (Iino et al., 2007; Le Roy et al., 2020), sugars (Otten et al., 2022) and lactate (Xu et al., 2024) respectively. I propose that the high abundance of these three genera in the wet, summer season is caused by the changes in prey diet causing changes in the lions by extension. Ungulates and grazers such as kudu (*Tragelaphus strepsiceros*) and impala (*Aepycerus melampus*) increase body masst between dry and wet seasons likely due to resource availability (Fuentes-Allende et al., 2023; Ogutu et al., 2015). Lions prey on these grazers and therefore consume more fats and sugars from their high body-mass prey. This increase in consumption may lead to an increase in fat and sugar degrading bacteria.

4.3. Novel genomes

The 272 newly generated MAGs contribute to current wild animal databases and expand gut metagenome comparisons given the absence of prior sampling or sequencing of the African lion gut microbiome. Metagenomic analyses are negatively affected by incomplete databases (de la Cuesta-Zuluaga et al., 2020), which can lead to incorrect conclusions drawn about the wild animal gut microbiome and can affect diversity metrics, beta diversity comparisons and abundance charts. The MAGs generated in this study can provide a significant contribution to current databases. Given that the majority of MAGs could not be classified to the species level and differ in average nucleotide identity (ANI) from current databases, they likely represent a variety of new species which could enrich current databases and aid in understanding of the functions of the lion gut microbiome in the future.

In view of the fact that none of the MAGs in the phylum Actinomycetota were classified to the species level, it is likely that there are species in this phylum unique to lions. More research of the lion gut microbiome could therefore contribute to understanding the phylum Actinomycetota. Interestingly, the phylogenetic distances between MAGs in this phylum were noticeably lower than other phyla, which could signify genomes from one or two novel species unique to the lion gut. A similar pattern seen in the phylum Bacillota_C supports this hypothesis; there were several closely related MAGs which were all classified to the species level and all belonged to the same species. The same is likely true that the closely related MAGs in the Actinomycetota section are all a part of the same unknown species. This trend of closely related species can be seen for the phyla Bacteroidota, BacillotaB and Pseudomonadota, indicating the possibility for multiple new species to be identified in MAGs generated from this research. At the genus level, the most number of MAGs were classified as *Collinsella* and *Fusobacterium_B* implying a similar addition to these genera in current databases as that for the phyla above.

4.4. Functional analysis

Functional analysis showed metabolism as the most abundant pathway, with carbohydrate and amino acid metabolism in highest abundance besides the generalised global and overview maps. Bacteria in the carnivore gut have a similar proportion of amino acid metabolism and carbohydrate metabolism genes (Zhu et al., 2018). This discovery of high carbohydrate metabolism is not

necessarily surprising as carbohydrates are common in the lion's diet (Borstlap, 2002). Carbohydrates can be ingested from prey tissues and plant matter consumed incidentally during feeding. The abundance of carbohydrate-metabolising genes suggests that lions metabolise carbohydrates as well as proteins for important energy production. Additionally, carbohydrate metabolism contains a diverse set of pathways with many enzymes and genes, thus this functional pathway may just be in higher abundance due to its higher number of 'possible hits' compared to amino acid metabolism with relatively fewer pathways ("KEGG PATHWAY Database," n.d.).

Looking at purine metabolism as a specific pathway, we can see that bacteria in the lion gut are able to metabolise purines to urea independently of the lion except for one step; the conversion of urate to 5-hydroxyisourate. In other words, none of the reads mapped to the gene for urate oxidase nor FAD-dependant urate hydrolase. This pathway was chosen specifically due to lions' high purine diet (Herring et al., 2021). Purine metabolism is essential for lions to effectively utilise dietary resources and maintain metabolic homeostasis. Purines are essential components of nucleic acids and play vital roles in cellular processes, making their metabolism crucial for cellular function and energy metabolism. The absence of genes encoding urate oxidase and FAD-dependent urate hydrolase in the lion gut microbiome may indicate bottleneck in purine metabolism if the lion is unable to bridge the enzymatic gap by compensating for these missing enzymes through endogenous pathways. Nonetheless, the presence of 18 purine metabolism genes is a good sign that the lions' gut microbiome is actively assisting it in an integral process to its health, once again emphasising the importance of these commensal bugs.

5. Conclusions

This study represents the first microbiome classification of African lions (*Panthera leo melanochaita*) as well as the largest microbiome study of lions to date. The most abundant bacterial genera present in the 23 African lion gut microbiome samples were *Bacteroides* and *Phocaeicola*, two genetically related genera from the same family. *Bacteroides* is the most abundant genus in the gut of other pack-hunting carnivores including black-backed jackals, wild wolves (Zhang and Chen, 2010) and dholes (Wu et al., 2022). Contrastingly, solitary hunting cheetahs have a high abundance of *Clostridium* (Menke et al., 2014; Wasimuddin et al., 2017) in their gut and a comparatively low abundance of *Bacteroides* (Menke et al., 2014). This may be attributed to a difference in bacterial gut composition between lions and cheetahs because of differences in their

social structures. Lions hunt together as a pride, whereas cheetahs typically hunt and consume prey individually (Hilborn et al., 2018). Pack-hunting animals are more likely to transfer bacterial species horizontally between individuals (Sarkar et al., 2020) and have different diets (Hayward and Kerley, 2008) compared to solitary hunters. t *Bacteroides* species in the gut may be tied to specific traits of pack-hunter carnivores.

The high abundance of *Bacteroides* in the lion gut was in contrast to the only previous study investigating lions specifically, which found *Fusobacterium* to be the most abundant genera in the gut microbiome of three Asiatic lion (*Panthera leo leo*) (Mittal et al., 2020). The three Asiatic lion samples were reclassified and analysed using the same classification tools alongside the African lions samples collected for this study and revealed a similar abundance of *Phocaeicola* to the African lions but a higher abundance of *Sutterella*. We attribute this differential abundance to allopatric separation between the two subspecies preventing transfer of microbial species and allowing for changes in the gut microbiome composition over time. Furthermore, we believe that since the genus *Phocaiecola* was similarly abundant in both subspecies, it serves a conserved function in the lion gut microbiome and is therefore an indispensable taxon. On the other hand, the genera *Bacteroides*, *Sutterella* and *Fusobacterium* varied in abundance between the two subspecies, suggesting non-conserved functions within the lion gut (Rojas et al., 2022).

The genus *Sutterella* was more abundant in female lions compared to male lions. *Sutterella* is linked to inflammation in the gut (Hiippala et al., 2016) and could therefore be related to a female-specific trait causing high inflammation. Pregnancy is a credible source of this inflammation and is further supported by a high abundance of *Sutterella* and *Fusobacterium* in one lion sampled post-pregnancy. Similar increases in *Fusobacterium* and inflammation have been seen in pregnant and lactating pigs (Liu et al., 2019) and primates (Hanning and Diaz-Sanchez, 2015; Sun et al., 2021). We therefore suggest a potential link between the presence of *Sutterella* and *Fusobacterium* and pregnancy in female lions. Further studies on the connection between pregnancy and the lion gut microbiome are needed to confirm these links.

Diet related links were also found between bacterial genera in the lion gut. The species *Plesiomonas shigelloides* was more abundant in the lion gut in winter compared to summer. This species is known to cause diarrhoea and food poisoning in mammals (Santos et al., 2015) and is most often transmitted through water sources. The increase in this pathogenic species could be

related to lions consuming more stagnant or unclean water during the dry winter when availability of water sources were limited. Monitor bacteria in water sources during the dry season may help to improve the health of lions and other carnivores.

Contrastingly, bacterial genera more abundant in summer indicated an increase in fat and sugar intake for lions during this season. The genera *Dysosmobacter*, *Clostridium* Q, *Oscillibacter* and *Metalachnospira* are related to metabolism and breakdown of fats (Roy et al., 2022), glucose (Iino et al., 2007; Le Roy et al., 2020), sugars (Otten et al., 2022) and lactate (Xu et al., 2024) respectively. With prey generally in better condition during wet summer months (Fuentes-Allende et al., 2023; Ogutu et al., 2015)may consume higher amounts of fats and sugars.

With regards to metabolism, carbohydrate and amino acid metabolism were the highest and most enriched pathways in the lion gut. Amino acid and carbohydrate metabolism are similarly represented in the carnivore gut microbiome (Borstlap, 2002; Zhu et al., 2018). The abundance of carbohydrate-metabolising genes implies that lions metabolise carbohydrates as well as proteins for important energy production. Within amino acid metabolism, that bacteria in the lion gut can metabolise purines to urea independently of the lion enzymes except for one step; the conversion of urate to 5-hydroxyisourate. Since the degradation of purines to uric acid is essential to the lion metabolism (Hou et al., 2020), lions likely have endogenous enzymes for all reactions in the uric acid degradation including the missing bacterial proteins urate oxidase and flavin adenine dinucleotide (FAD)-dependant urate hydroxylase The heavy contribution of bacterial genes to purine metabolism emphasises the importance of commensal microorganisms in the mammalian gut.

There was a very high proportion of unclassified reads using different classification methods: MetaPhlAn, GTDB, MAG comparisons and Kraken2. All four of these tools (with corresponding databases) classified at most 70% of the reads. However, with the last tool, Kraken2, reads from the lion gut microbiome were aligned to the *nt* database; a large database containing genomes from bacteria, protozoa, fungi, plants and mammals (Wood and Salzberg, 2014). Around 30% of the reads were still unclassified on average. We are therefore certain that these unclassified reads represent new species of microorganisms not present in current databases thus emphasising the need to contribute to existing databases especially with sequences sampled from unique or neglected environments such as the lion gut microbiome (Nayfach et al., 2016).

This study identified 272 MAGs which will contribute significantly to current databases. Given that the majority of MAGs could not be classified to the species level and differed in average nucleotide identity by more than 95% from current databases, they represent new species not present in current databases. Further analysis of these MAGs will help to identify specifics about these new species and support the development of current databases. To further aid this advance, DNA extracted from the lions sampled in this study has been sent for Oxford Nanopore Technologies (ONT) long read sequencing. The output of long read sequencing will be helpful in identifying the function and phylogenetic classification of these unknown MAGs in a future study.

6. Future research

Although the sample size of 20 lions used in this study is the largest to date, future studies including a larger cohort of lions would be useful to confirm some of these findings. Firstly, a larger sample size would increase the statistical power to find and confirm differences between demographic, such as age and sex, and environmental variables influencing the gut microbiome of lions. Secondly, more in depth monitoring tools such as blood sample testing or diet monitoring could also help place the findings in context and draw stronger correlations between bacterial species and inflammation or disease. For example, a study monitoring carnivore's hunting activity before collection of microbiome samples could help to draw specific connections between diet and bacterial species. Similarly, expanding these investigations to captive lions would help to confirm connections between the gut microbiome and factors that might be more difficult to monitor in the wild. For example, it would be difficult to capture a lion in the wild during and after pregnancy to collect faecal samples. Monitoring captive lions would help to confirm the links between inflammation and pregnancy.

By the same token, expanding microbiome studies to more species will help to test hypotheses made around carnivores in general A study comparing the gut microbiome of solitary and pack hunting carnivores would be insightful from an ecological perspective to confirm that these two hunting strategies influence the gut microbiome. Furthermore, investigations into the consumption of Lepidoptera and fish not typically investigated in traditional dietary studies could provide insights to microbiome classifications. For example, careful monitoring of the diet of lion prey before they are consumed by lions could identify if lions incidentally consume insects indirectly through their prey.

As databases are improved and added to, the data from this study could be taken and reanalyzed. As was clear from this study, reanalysing samples years later can yield significant different results. Thus the observations made here will become more robustas databases grow. In the same vein, resequencing the samples collected in this study will expand the findings here. The samples have undergone Oxford Nanopore Technology (ONT) sequencing and steps to analyse this data are currently underway.

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8. Appendix

8.1. International shipment of samples from Namibia to South Africa.

A note on the order of permits:

There are two CITES applications required for shipment - a Namibian CITES export permit and a South African CITES import permit. The Namibian CITES export permit application states that it requires a South African CITES permit in the application. Upon applying for a South African CITES import permit however, authorities requested a Namibian CITES permit first. It is still confusing as to which permit is required first. In our case, we got our Namibian CITES first and then applied for the South African CITES.

From the information I could gather, it seems that for species in Appendix I (e.g. leopard, tigers, black rhino, cycads), an import permit is required for an export to be issued. Lions are in appendix II, which requires an export first followed by an import permit. Why the order changes for the two appendices is unknown.

This chain of permits was a problem throughout our applications - as you can see below many permits are required for other ones and it is difficult to know where to start.

A note on feedback:

The below estimated times do not include any misinformation on the permits. There were many times where I missed a section or filled something in incorrectly - the permit office will usually not let you know of any of these mistakes but will also just leave the application if it is not correct. Always make sure to follow up.

Our worst case was the Namibian CITES export permit which took about 4 months to issue. The permit was then collected by an unknown person - I think that the office gave it to someone by mistake - and we had to apply for it over again.

Other times information was not provided. When applying for our veterinary import permit for pathology specimens we requested a road border to be used. About a month after this submission of the application I eventually requested the number of the state vet assigned to our permit and called her. Only then was it explained that some ‘high-risk’ samples can only be imported via flight because it is more controlled. We never would have known this unless I had called the specific vet assigned to our case as the permit office did not inform us even after calling. We also would have then had to re-apply with the new changes and wait all over again. Luckily I was able to email the amended application directly to the state vet.

Namibian permits:

National Commision on Research, Science & Technology (NCRST) permit for non-Namibian based research institute/person: this permit is required for collection of the samples in Namibia. It is also required for export of the samples. In my case this was valid while the samples were collected however expired by the time the samples left Namibia. I added an affidavit stating that the samples were collected while the permit was valid as well as an email from the NCRST stating that it is okay to export the samples under the expired permit.

Namibian CITES export permit: CITES regulations differ between countries and based on the category of the animal. In the case of lions, they are currently in appendix II - Namibia requires a CITES permit in this case but South Africa does not. This permit took the longest of all the others and was also closest to expiring when I shipped the samples. To apply for it you must fill in a hard copy application and drop this off at the Ministry of Environment, Forestry and Tourism (MEFT) in Windhoek. This application states that it requires a South African CITES permit in the application, however we did not have one when applying (please see above). It must also be picked up from MEFT and the original must be included in the shipment. In the case of lions, South Africa did not require a CITES permit for import but Namibia did and so I had to apply for both regardless.

~ 6 months. Contact: CITES namibia - +264 61 284 2546 - K Katjimune - katjimune.kambangu@gmail.com

South African permits:

South African CITES import permit: see notes above on CITES permits. This can be applied for via email and through Wits. The order in which these permits are required is still unknown as explained above. The original must be included in the shipment and can be collected from Environment House in Pretoria.

~ 8 days. Ntwanano - NMasingi@environment.gov.za

Section 20 permit: this permit can be applied for via email. This requires a letter from the state veterinarian in Namibia stating whether the samples are under any disease restrictions as well as a certificate of registration from the disposal company responsible for anything that happens to your samples in South Africa (in our case this was Buhle Waste).

~ 3 months including collection of required documents. Miss Marna Laing - MarnaL@dalrrd.gov.za. You will be assigned an official

Application to import pathology specimens and raw materials for laboratory use (Vet permit): your application will be assigned to a state vet - if absolutely necessary you can request their contact details. This permit application requires a section 20 permit - see above. This can be applied for via email but must be collected in person from the Delpen Building in Pretoria. The original must be included in the shipment. This permit requires a Namibian state vet to be on site when the samples are packaged. I have included an extensive list of Namibian state vets and their

locations below In our case, the closest state vet to Etosha is in Outjo (Dr Kazarako). Note that samples cannot cross the red line without being packaged in front of a veterinary official. The procedure I would recommend would be to bring the samples to Anderson Gate and request that the Outjo vet meet you there. You can then package the samples in front of them as per red line regulations and sign the vet letter at the same time. Packaging the samples in this way requires a security seal shown below:

These seals can be bought from Agra in Outjo. The shipping company should also send you packaging that includes a biohazard bag and a shipment box within a cardboard box. The cardboard box can be sealed as shown above.

~ 1 month. VetPermits@dalrrd.gov.za - Dr Gretna de Wit - GretnaDW@dalrrd.gov.za

In summary you will need:

- Section 20 permit (SA) - Requires a vet letter as well as a waste disposal registration certificate.
- Vet permit - Requires section 20 permit. Original.
- Namibian CITES export permit - Original.
- South African CITES import permit - Original.
- NCRST permit - can be expired but may need supporting documents.
- I would recommend you apply for the permits in the order above (excluding the NCRST permit which you should already have for sample collection).

Namibian state vets:
Outjo
Dr Ndjoze (no longer at Outjo)
Dr Kazarako
+264 81 1291034

+264 67313050
Kamanjab
Dr Katumbe
+264 82 472 6163
Windhoek
Windhoek state veterinary office
+264 61-27-6580
Dr Murangi
+264 81-660-3048
Dr Frans Alugongo (falugongo@yahoo.com)
+264 81 5684870
Otjiwarongo
Dr Uusiku
+264-67-302131
Central Veterinary Laboratory
+264-61-276-580

8.2. Nanodrop DNA concentration results

Table S1: Concentrations of DNA extracted from samples collected from lion microbiome samples in Etosha National Park, Namibia. Yellow highlight indicates samples that were sent for shotgun sequencing below 10 ng/µl, green highlight indicates which of the duplicate extractions was sent for each sample and red outline indicates extractions below the recommended 10 ng/µl.

Sample	Extraction 1 concentration (ng/µl)	Extraction 2 concentration (ng/µl)
F1	5.77	10.308
F2	2.469	4.498
F3	37.28	48.07
F4	18.767	36.489
F5	11.356	11.571
F6	16.713	29.923
F7	16.313	17.707
F8	70.722	42.411
F9	4.587	1.772
F10	15.445	17.939
F11	11.026	25.114
F12	16.501	6.352
F13	24.654	17.771
F14	9.562	11.314
F15	39.802	45.166
M1	9.034	
M2	28.951	22.733
M3	10.72	6.547
M4	6.488	12.417
M5	13.499	48.735
M6	22.705	12.144
M7	39.107	2.7
M8	1.281	1.393

8.3. MultiQC results

Table S2: MultiQC results after pre-processing of forward reads sequenced from the gut microbiome of lions in Etosha National Park, Namibia.

Sample	Duplicates (%)	GC content (%)	Read Length (bp)	Million Sequences
F1	29.80	46	146	6
F2	30.10	43	147	6.6
F3	20.00	47	141	5.7
F4	13.90	43	147	1.7
F5	25.50	45	145	5.7
F6	22.60	45	145	6.3
F7	21.80	47	145	6
F8	31.30	42	147	8.3
F9	16.60	35	146	1
F10	24.20	48	144	4.9
F11	25.30	43	147	5
F12	11.10	41	147	3
F13	14.30	37	148	2.2
F14	12.90	47	146	6.3
F15	11.40	46	146	6
M1	13.90	39	148	3.7
M2	19.70	42	146	2.6
M3	15.40	43	148	7
M4	23.40	47	146	8.2
M5	11.70	45	147	2.2
M6	21.00	49	145	7.6
M7	15.20	46	147	3.2
M8	19.80	46	146	3.7

Table S3: MultiQC results after pre-processing of reverse reads sequenced from the gut microbiome of lions in Etosha National Park, Namibia.

Sample	Duplicates (%)	GC content (%)	Read Length (bp)	Million Sequences
F1	29.70	46	146	6
F2	29.90	44	146	6.6
F3	20.10	47	141	5.7
F4	14.00	43	147	1.7
F5	25.50	45	145	5.7
F6	22.70	45	145	6.3
F7	21.80	47	144	6
F8	31.00	42	147	8.3
F9	16.80	35	146	1
F10	24.20	48	144	4.9
F11	25.10	43	147	5
F12	11.20	41	147	3
F13	15.00	37	148	2.2
F14	13.10	47	145	6.3
F15	11.30	46	146	6
M1	13.60	39	148	3.7
M2	20.20	42	146	2.6
M3	15.20	43	147	7
M4	23.40	47	145	8.2
M5	11.80	45	146	2.2
M6	21.00	49	145	7.6
M7	15.00	46	147	3.2
M8	19.80	46	146	3.7

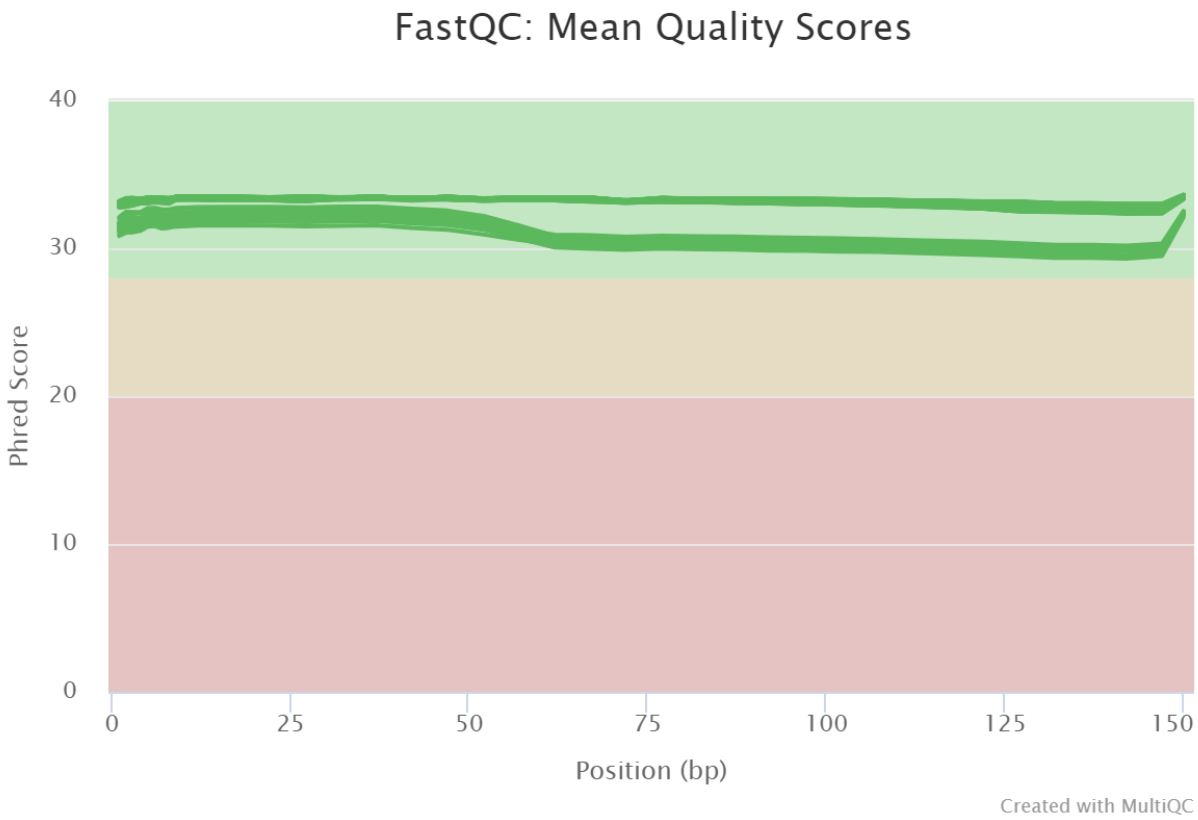

Figure S1: Mean quality variation across each base pair in a read for all pre-processed reads sequenced from the gut microbiome of lions in Etosha National Park, Namibia.

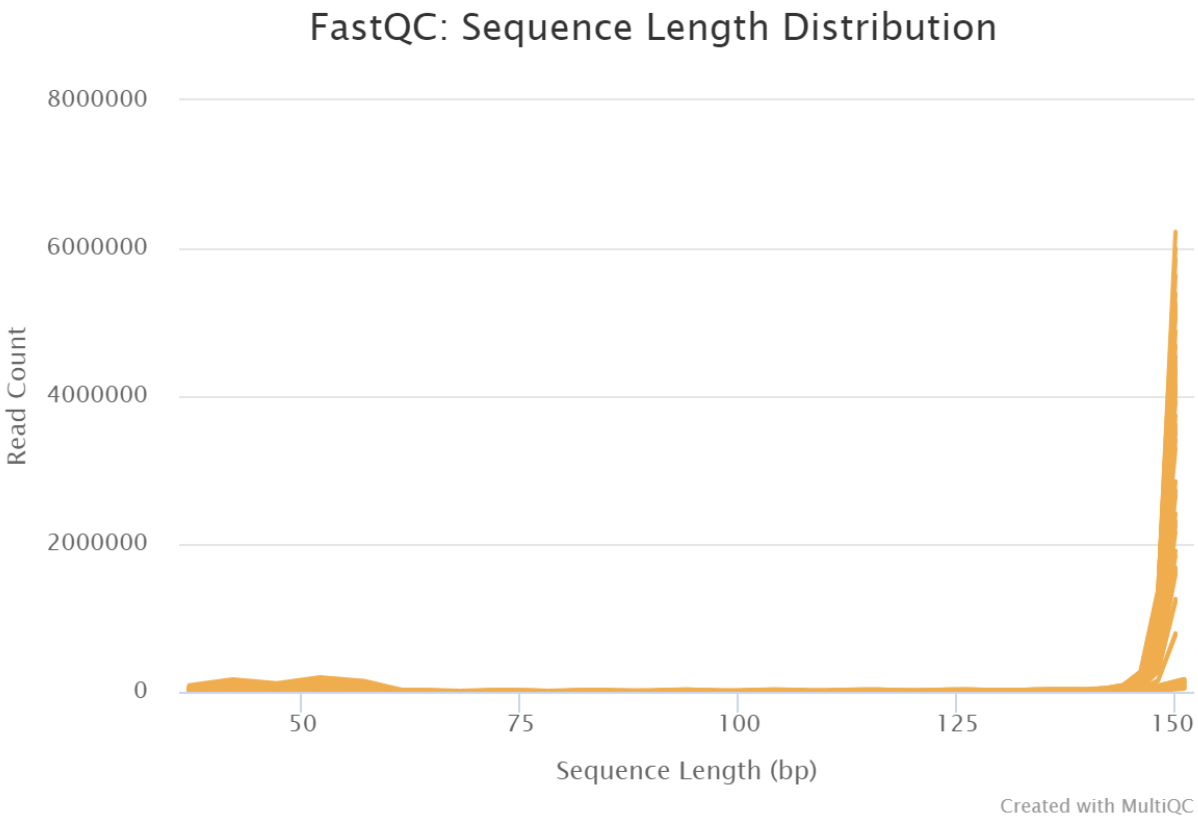

Figure S2: Distribution of read length. The number of reads of each length for pre-processed reads sequenced from the gut microbiome of lions in Etosha National Park, Namibia.

8.4. Excluded South African lion samples

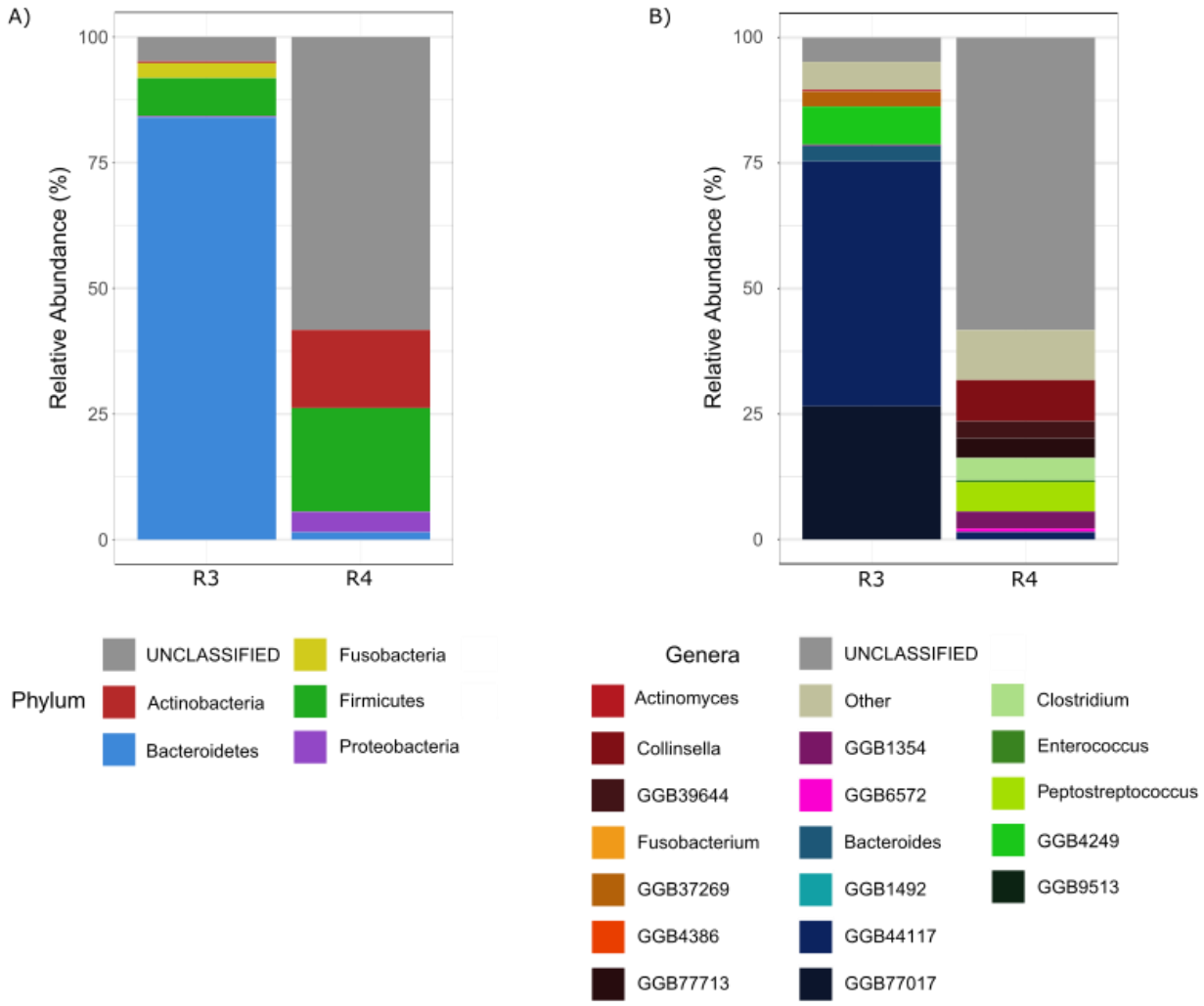

Figure S3: The lion gut microbiome of lions sampled in South Africa. Bar plot showing the relative abundance of **A)** bacterial phyla and **B)** bacterial genera present in the gut microbiome of excluded samples from lions in South Africa using MetaPhlAn classification. Genera are coloured by phylum using the colours shown in panel A.

8.5 Metagenome-assembled genomes quality

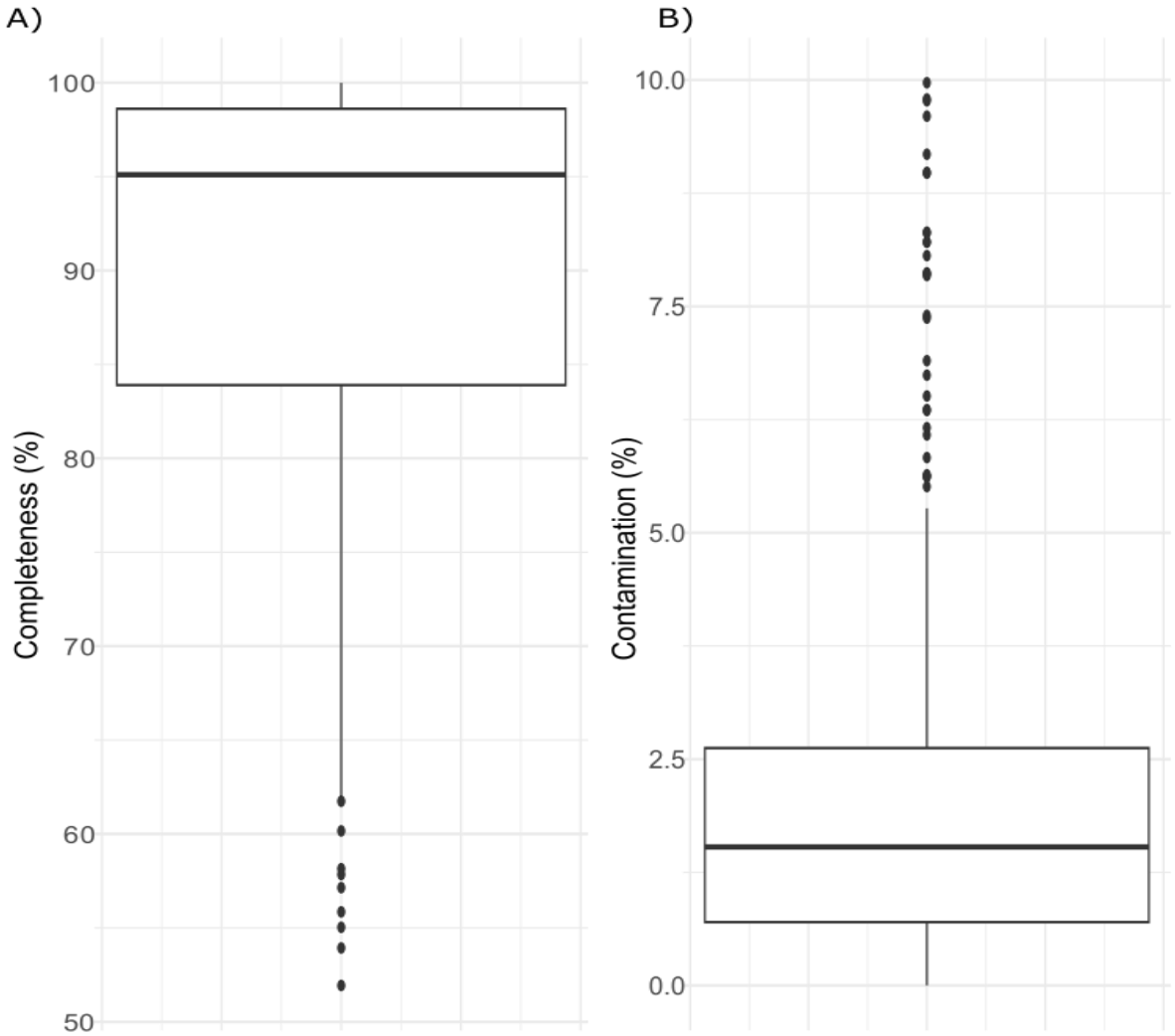

Figure S4: Completeness and contamination for 272 MAGs created from whole metagenome sequenced reads from the lion gut microbiome. A) Box plot showing percentage completeness for all MAGs. B) Box plot showing % contamination of all MAGs.