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Investigating the effects of *Mycobacterium suricattae* infection on the gut microbiome of wild meerkats (*Suricata suricatta*) applying High Throughput Sequencing technology

University of Ulm

Faculty of Natural Sciences

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Bachelor Thesis

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STATEMENT OF AUTHORSHIP

I declare that this thesis was authored independently and that only the declared sources were used. All literally or by content quoted sources were marked explicitly. Furthermore, I declare that the thesis has not been submitted to any other examination board.

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(Place, Date) (Signature)

Supervised by:

Prof. Dr. Simone Sommer

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Sincerely,

Martin Geiger

ABSTRACT

The *Mycobacterium tuberculosis* complex (MTBC) contains distinct lineages of zoonotic pathogens causing tuberculosis (TB) disease in humans and a variety of wild and domestic mammal species, including meerkats (*Suricata suricatta*). One mechanism by which TB may affect host health is via its effects on the gut microbiome, because this community of microbiota is critical for host function. Stressors such as TB infection may cause microbiome dysbiosis, whereby microbiome composition shifts, potentially permanently, into a state that promotes pathogenesis in the host. TB infection may cause dysbiosis via two mechanisms: 1) infection induces a deterministic shift into an alternative stable state; 2) infection has stochastic effects on the microbiome, creating an unstable community configuration with greater dispersion (the "Anna Karenina principle"). Alterations to the gut microbiome composition induced by TB infection are insufficiently investigated in numerous species. In this thesis, I test for the effects of infection on the gut microbiome using TB and meerkats as model system. Meerkats are small carnivores inhabiting desert regions of southern Africa, and are routinely affected by outbreaks of meerkat TB (*Mycobacterium suricattae*). I analyzed 16S amplicon microbiome data from 362 samples collected from 58 individuals with known TB infection history. I found that *M. suricattae* infection is associated with negative effects on physical condition of meerkats. However, no alterations in alpha or beta diversity of gut microbiome composition linked to TB infection or symptoms were detected. The findings reveal the difficulty to identify drivers of microbiome dynamics in meerkats due to natural variation and temporal fluctuations. Nevertheless, TB infection may affect specific strains, which was not tested for in this thesis. Further experimental studies may unravel the highly complex responses of host factors, including gut microbiome, to TB infection, potentially providing possibilities for preservation of wildlife species and minimizing the risk of this disease on public health.

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LIST OF ABBREVIATIONS

AIC	Akaike information criterion
ASV	Amplicon sequence variant
DADA 2	Divisive Amplicon Denoising Algorithm 2
DNA	Deoxyribonucleic acid
GLMM	Generalized Linear Mixed Model
ID	Identification
MTBC	*Mycobacterium tuberculosis* complex
OTU	Operational taxonomic unit
PCoA	Principle coordinates analysis
PCR	Polymerase chain reaction
PD	Phylogenetic diversity
PERMANOVA	Permutational multivariate analysis of variance
QIIME 2	Quantitative Insights Into Microbial Ecology 2
TB	Tuberculosis
TS	Target specific

1 INTRODUCTION

Tuberculosis (TB) is an infectious disease occurring in a variety of mammalian hosts. It is caused by genotypically and phenotypically distinct lineages of the *Mycobacterium tuberculosis* complex (MTBC) (Dippenaar et al., 2015). *M. tuberculosis* infections are prevalent in about a quarter of the human population and led to 1.5 million deaths in 2018, thus being responsible for the most deaths caused by a single infectious agent (World Health Organization, 2019). Since several members of the MTBC affect both humans and numerous animal species, both domestic and wildlife, these zoonotic pathogens constitute a major problem for public health programs, as well as for wildlife conservation (Alexander et al., 2002; Clarke et al., 2016). Notably, wildlife disease research over the last decade has identified TB as a major threat to various wildlife populations, including European badgers (*Meles meles*)(Cheeseman et al., 1989), the African buffalo (*Syncerus caffer*) as the key reservoir species in Africa (Renwick et al., 2006), and the Australian brushtail possum (*Trichosurus vulpecula*) being considered the main reservoir in New Zealand (Ryan et al., 2006). In some cases, disease epidemics are caused by *Mycobacterium bovis*, transmitted by domestic cattle acting as spillover host (Palmer et al., 2012). Other species host their own mycobacterial species, for example banded mongooses (*Mungos mungo*), which are infected by *Mycobacterium mungi* (Alexander et al., 2010), and rock hyraxes (*Procavia capensis*), which are associated with the dassie bacillus (Mostowy et al., 2004). However, while required for effective control of TB, the pathogenesis and epidemiology of *Mycobacterium* strains in wildlife populations is still poorly understood, as interactions between hosts, pathogens, and environment are highly complex (Drewe et al., 2009; Ryan et al., 2006).

One mechanism by which TB infection may affect host health is via changes in the gut microbiome (Luo et al., 2017). The term microbiome refers to the entire community of microbiota, belonging to all three domains of life (Bacteria, Archaea and Eukarya), occupying the skin and all mucosal surfaces of the body (Dumas et al., 2018). The gut microbiome provides numerous functional benefits to the host, including nutrient breakdown and synthesis of vitamins and co-factors (O'Toole, 2012), growth limitation of opportunistic pathogens via resource competition (Wood et al., 2017), as well as regulation of development and homeostasis of innate and adaptive immune cells (Hill & Artis, 2010). Microbiome composition remains in a robust homeostatic equilibrium in healthy individuals (Das & Nair, 2019). However, perturbations such as antibiotics or infections can disrupt microbial homeostasis and induce long-term alterations in microbiome composition, termed 'dysbiosis' (Zaneveld et al., 2017).

Microbiome dysbiosis can affect host health negatively by depleting beneficial symbiotic strains and increasing the abundance of opportunistic or pathogenic strains, and is therefore associated with health disorders like malnutrition and metabolic disorders (Chng et al., 2020; Das & Nair, 2019). Recovery of the microbiome back to its original state may be facilitated by synergistic interactions of certain bacterial strains that promote community resilience (Chng et al., 2020).

The stress caused by pathogen infection can induce microbiome dysbiosis via two major mechanisms (Zaneveld et al., 2017), which are illustrated in Figure 1. Firstly, infection can shift the composition of the gut microbiome into an alternative stable state, whereby microbiome alpha (within individual) and beta (between individual) diversity are altered in similar, predictable ways in response to equal perturbations. Resulting alternative stable state communities between infected and uninfected individuals are similar in dispersion and identical in beta diversity, and alpha diversity may either be higher or lower in infected versus uninfected individuals. Secondly, alterations in microbiome composition due to perturbations can be stochastic, rather than deterministic, and cause stable communities to become unstable, leading to higher variation in both alpha and beta diversity in infected versus uninfected individuals. Adaptation of Leo Tolstoy's dictum that “all happy families look alike; each unhappy family is unhappy in its own way” to microbial ecology resulted in the “Anna Karenina principle” describing microbiome dynamics in animals. It is argued that effective regulation of microbiome composition is reduced or breaks down due to perturbations, which in turn leads to a shift from similar healthy stable states to a wide range of dysbiotic unstable community configurations, where every dysbiotic microbiome is distinct. These perturbation induced stochastic processes lead to gradually increasing beta diversity from healthy to stressed hosts, depending on stress severity (Zaneveld et al., 2017). Finally, it should be noted that deterministic and stochastic influences on microbiome composition are often interconnected, for example when perturbations alter the microbial community deterministically towards an alternative stable state, but to a stochastic extent based on stressor severity (Stegen et al., 2012; Zaneveld et al., 2017). In this case beta diversity in mildly stressed hosts is high, while it is low in healthy and severely stressed hosts (Zaneveld et al., 2017).

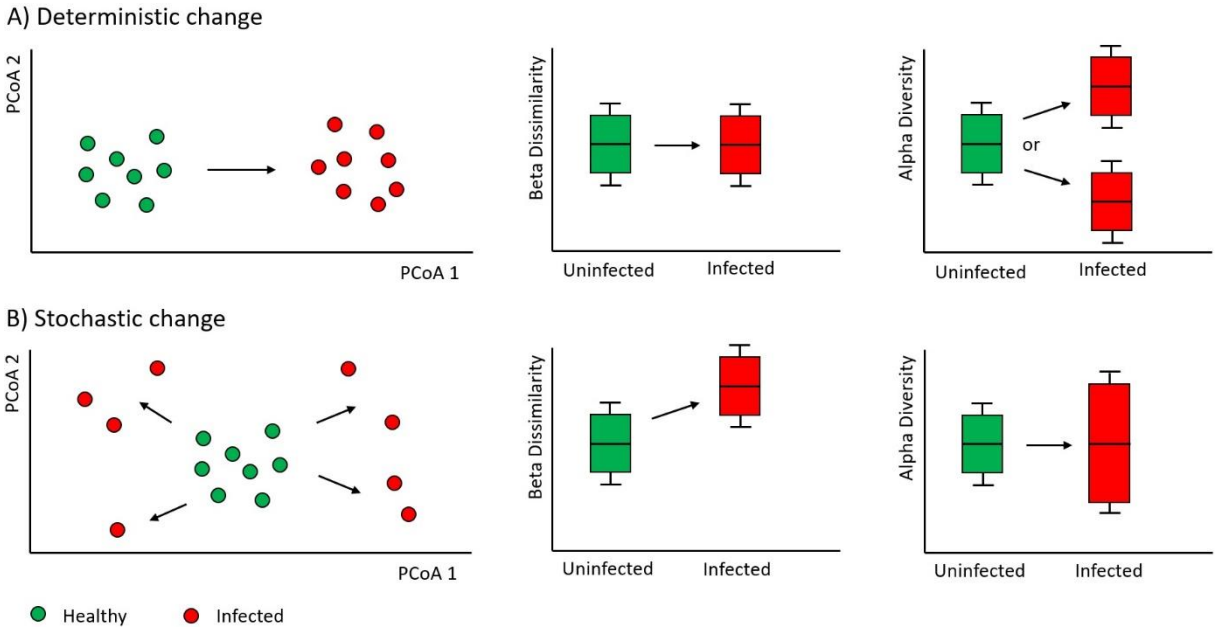

Figure 1: Representation of hypothetical alterations in microbiome composition due to perturbations induced by infection. Microbiome composition is marked green for healthy individuals and red for infected individuals. A) Deterministic change shifts microbiome into an alternative stable state. Alpha diversity may be either higher or lower, beta diversity within each group is identical, and dispersion is similar. B) Stochastic change leads to unstable communities and higher variation in alpha and beta diversity in infected individuals, increasing with stress severity.

Evidence for these two major mechanisms are limited and mixed (Zaneveld et al., 2017), and different pathogens may have different effects on the microbiome (Duvallet et al., 2017). A deterministic shift into an alternative stable state was reported after initial microbial diversity loss caused by TB infection in mice (Winglee et al., 2014). Arising six days after infection via aerosols, the pathogen led to a rapid decrease in overall community diversity. Despite a recovery in microbial diversity until death, microbiome composition remained significantly different afterwards. The authors posit that the loss of diversity resulted from host immunological changes and diversity recovered as soon as an equilibrium between bacterial burden and adaptive immune activity was reached (Winglee et al., 2014). Evidence for a stochastic change was provided by a study investigating gut microbiome alterations in patients with active pulmonary TB (Maji et al., 2018). Despite a decrease in *Prevotella* and *Bifidobacterium* abundance in TB patients compared to their healthy household contacts, increased microbial diversity was observed, characterized by enrichment of bacteria with pathways for butyrate and propionate production like *Faecalibacterium*, *Roseburia*, *Eubacterium* and *Phascolarctobacterium*. This dysbiosis resulted in an abrupt increase in butyrate levels, which was associated with an impaired host immune response towards TB infection (Maji et al., 2018). A significant decrease of *Prevotella* in new and recurrent TB patients was detected by another study as well (Luo et al., 2017).

Further observations included a decrease of the most abundant phylum Bacteroidetes, which contains various beneficial symbiotic bacteria, in new and recurrent TB patients, and a significant increase of Actinobacteria and Proteobacteria, known to comprise numerous pathogenic species, in recurrent TB patients. In comparison to the control group, alpha diversity was moderately increased in new, and significantly increased in recurrent TB patients, and variation in beta diversity was significantly increased in both groups (Luo et al., 2017). In summary, both major mechanisms were observed in previous studies, more evidence however can be found for stochastic changes of gut microbiome composition.

In this thesis I aim to test for the effects of infection on the gut microbiome using TB in wild meerkats (*Suricata suricatta*) as a model system. Meerkats are small, diurnally active carnivores that belong to the family Herpestidae and inhabit open thorn and grassland savanna in arid and semiarid regions of southern Africa (Jordan et al., 2007; Moss et al., 2001). Cooperative behavior of meerkats, living in social groups of 2-49 individuals, causes transmission of infections within their population (Jordan et al., 2007; Patterson et al., 2017). The first outbreak of TB in free-living meerkats has been reported in the Kalahari Desert, South Africa, in 2002 (Alexander et al., 2002). The organism causing TB in meerkats was later identified as a novel, genetically unique strain of the MTBC, called *Mycobacterium suricattae* (Parsons et al., 2013). Transmission of TB occurs by inhalation of aerosols from individuals with active TB, but can also result from biting (Clarke et al., 2016). Successful infection leads to latent TB infection which eventually might progress to active TB disease (Namasivayam et al., 2018). Latent infection can last years or even decades, both in humans (Lillebaek et al., 2002) and wildlife (badgers: Cheeseman et al., 1989; elephants: Obanda et al., 2020). This has interesting implications for the interaction between infection and gut microbiome composition, since TB infection occurs in two distinct states (latent and active). Importantly, once infected with TB, it is widely assumed that animals are infected for life unless they undergo medical treatment. As such, studies on TB in wildlife generally follow an epidemiological 'system of no return', in that one animal is found to be infected, it is assumed infected for life, even if subsequent tests are negative (McDonald et al., 2019).

The main goal of this study was to investigate the effects of TB infection on gut microbiome composition in wild meerkats. I analyzed longitudinal gut microbiome data from 362 fecal samples belonging to 58 individuals, for which TB infection and symptom status was known. This data, including samples from before TB infection, allowed me to follow how TB is associated with gut microbiome composition over time within the same individuals.

Since the meerkats' physical condition may mediate associations between TB and the gut microbiome, I first investigated possible relations of TB infection and TB symptoms to condition. An additional analysis of the influence of a number of biological and methodological variables was carried out. Specifically, I tested the following hypotheses:

I. Uninfected meerkats are in better physical condition than infected meerkats.

IIa. Variation in alpha diversity is higher in infected versus uninfected samples if effects of TB infection are stochastic.

IIb. Because latent infections often do not affect host health, samples taken from TB symptomatic meerkats may therefore vary more in alpha diversity than those from non-symptomatic individuals.

IIIa. Dispersion in beta diversity is higher when meerkats are infected versus uninfected, according to the Anna Karenina principle.

IIIb. Samples taken when an individual is non-symptomatic may be more similar in their beta diversity to uninfected than infected states, since latent infections are often undetectable and with little effect on host health.

2 MATERIALS & METHODS

Fecal sample collection, DNA extraction and polymerase chain reaction (PCR) tests for TB were carried out in advance to this study. My contribution in this thesis was to analyze microbiome composition in relation to meerkat metadata, including TB infection status. Below I briefly describe all laboratory and bioinformatic steps necessary to replicate this study. I directly contributed to 2.3 (Library preparation) and 2.5 (Data analysis).

2.1 SAMPLE COLLECTION AND TB INFECTION

Meerkat fecal samples were collected from free-living meerkats at the Kalahari Meerkat Project, located in the Kuruman River Reserve (26°58′ S, 21°49′ E) in South Africa (Patterson et al., 2017). This population has been studied in depth since 1995 and therefore life history data for most individuals is known, including date of birth and death, sex, group membership, as well as their age and weight at sampling (Drewe et al., 2009). Notes were also taken regarding TB symptoms, including visibly enlarged submandibular and medial retropharyngeal lymph nodes (Patterson et al., 2017). The meerkats were individually recognizable and habituated to close observation (Jordan et al., 2007), allowing for opportunistic collection of fecal samples rapidly upon observing defecation (Moss et al., 2001). Samples collected before 2008 were stored frozen, whereas samples collected after 2008 were freeze-dried. In this study 362 samples from 58 individuals belonging to 23 different social groups, collected between 09/2000 and 09/2018, were analyzed.

2.2 DNA EXTRACTION AND AMPLIFICATION

DNA extraction from fecal samples of meerkats was carried out according to the manufacturer's instructions by using the NucleoSpin 96 Soil extraction kit (Machery-Nagel, Düren, Germany). The DNA extracts were subsequently screened for TB by PCR amplification, using primers for a partial sequence of the insertion sequence IS*6110*. Each sample was tested up to ten times, or until the first positive result was recorded. Once a sample was found positive, the meerkat was assumed infected for life following McDonald et al. (2019).

2.3 LIBRARY PREPARATION

In order to generate an Illumina MiSeq DNA Amplicon sequencing library, a fragment (291 base pairs (bp)) of the hypervariable V4 region of the 16S rRNA gene was amplified by using the universal bacterial primers 515F (5'-GTG CCA GCM GCC GCG GTA A-3') and 806R (5'-GGA CTA CHV GGG TWT CTA AT-3')(Caporaso et al., 2010, 2011). A 2-step PCR was carried out following the 4-primer amplicon tagging scheme of Fluidigm (Access Array System for Illumina Sequencing Systems, Fluidigm, San Francisco, USA). For the target specific (TS) PCR the primers were appended with either a CS1 adapter (CS1-515F) or CS2 adapter (CS2-806R). Four random bases were added to the forward primers (CS1-NNNN-515F) to avoid errors during cluster identification on the Illumina MiSeq. During the barcoding PCR a sample-specific barcode (10 bp) and adapter sequences required for Illumina sequencing were added to the CS1 and CS2 adapters.

The target specific (TS) PCR contained 2.50 µL H_2O, 5.00 µL Taq DNA Polymerase (AmpliTaq Gold 360 Master Mix, Thermo Fisher Scientific, Waltham, USA), 1.50 µL TS primer mix (200 nM each primer), and 1.00 µL template DNA (5-10 ng DNA), adding up to a total volume of 10.00 µL. The PCR was performed on a SimpliAmp Thermo Cycler (Thermo Fisher Scientific) with an initial denaturation at 95 °C for 10 min, 30 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 45 s, followed by a final elongation at 72 °C for 7 min. Successful amplification was confirmed by gel electrophoresis. Therefore 2 µL loading dye and 3 µL PCR product were loaded onto a 1.5 % agarose gel in TAE buffer next to a DNA ladder, and run at 110 V for 30 min. The subsequent barcoding PCR contained 4.00 µL H_2O, 10.00 µL Taq DNA Polymerase, 4.00 µL of unique barcode for each sample, and 2.00 µL of TS PCR product, adding up to a total volume of 20.00 µL. Apart from a reduction to 10 amplification cycles the PCR was performed equally to the first PCR.

The amplified samples were cleaned up by using the NucleoMag NGS Clean-up and Size Select Kit (Machery-Nagel, Düren, Germany) and quality checked on a random basis by capillary electrophoresis (QIAxcel Advanced, QIAGEN, Hilden, Germany). Afterwards sample concentrations were measured with picogreen fluorescence on a Tecan Infinite 200 Pro (Tecan, Crailsheim, Germany) and the samples were normalized to equimolar DNA amounts in the final pool. The library was prepared according to Illumina recommendations (Denature and Dilute Libraries Guide 15039740) with a final concentration of 8 pM and the flow cell was loaded onto the Illumina MiSeq for paired-end sequencing.

2.4 BIOINFORMATICS

The FASTQ files originating from the Illumina run were processed with "Quantitative Insights Into Microbial Ecology 2" (QIIME 2, Version 2020.2)(Caporaso et al., 2010). After trimming the primers, the forward reads were truncated at 247 bp and the reverse reads at 237 bp. The "Divisive Amplicon Denoising Algorithm 2" (DADA2) pipeline was used for quality filtering, merging, and chimera removal (Callahan et al., 2016). While other methods for quality filtering are constructing operational taxonomic units (OTUs) by clustering sequences according to a fixed dissimilarity threshold, DADA2 makes use of the quality of Illumina sequencing and resolves amplicon sequence variants (ASVs) that differ by only a single nucleotide (Callahan et al., 2016). Taxonomy was assigned using the SILVA database (Version 132)(Quast et al., 2013). FastTree was used to create a phylogenetic tree for the remaining ASVs (Price et al., 2010). An archaeon sequence was utilized to root the tree and was subsequently removed. Mitochondria and chloroplasts were removed within R (Version 4.0.2).

2.5 DATA ANALYSIS

Samples were normalized by rarefaction (to 8,000 reads; random seed set to 100 for replication purposes) to control for variation in sequencing depth (McKnight et al., 2019; Figure 2). Five samples were discarded at this point due to low sequencing depth, leading to 362 samples from 58 individuals in the final dataset. 1,341 ASVs were removed after random subsampling.

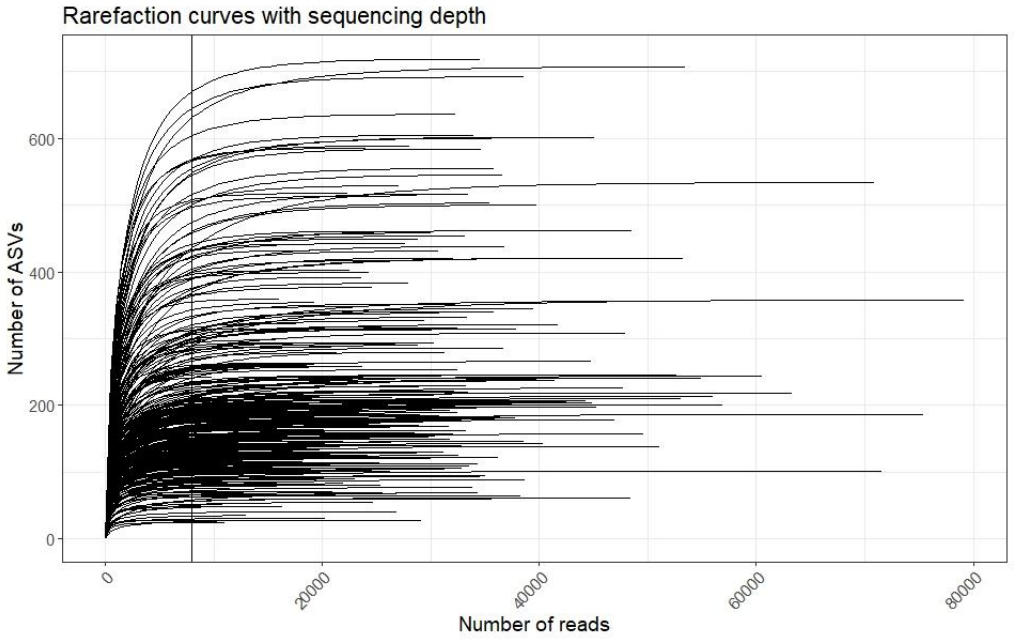

Figure 2: Rarefaction curves, showing the number of ASVs plotted against sequencing depth. Rarefaction cutoff threshold was set to 8,000 reads.

Because associations between TB and the gut microbiome may be mediated by meerkat physical condition, I first looked at whether TB infection (according to PCR) and TB symptoms were related to condition. Physical condition was represented by the residuals from a linear regression model predicting age against weight. Individuals exhibiting higher weight than predicted for their age were considered in good condition, whereas individuals exhibiting lower weight than predicted for their age were considered in bad condition. I performed a Generalized Linear Mixed Model (GLMM) with a Gaussian distribution, to test for differences in condition between TB uninfected and infected samples, and TB non-symptomatic and symptomatic samples. The model contained individual identification (ID) as a random variable in order to account for pseudo-replication (Jamil et al., 2013).

I next tested for differences in gut microbiome alpha and beta diversity between TB uninfected and infected, as well as non-symptomatic and symptomatic meerkats, whilst controlling for a number of methodological (sequencing depth and storage method) and biological (sex, physical condition, age at sampling, days until death) variables. While rarefied data accurately represents the original communities (McKnight et al., 2019), effects of sequencing depth may occur nevertheless.

2.5.1 Alpha diversity

Alpha Diversity is defined as the intra-community diversity, quantifying the number of individuals within a habitat or per sample (Thukral, 2017). In this thesis, I tested associations between TB infection and three measures of alpha diversity – observed richness, Shannon's diversity index, and Faith's phylogenetic diversity (PD). Observed richness is the total number of species (here ASVs) detected in a sample, or in a set of samples (Gotelli & Colwell, 2011). Shannon diversity takes relative ASV abundance into account, i.e. levels of dominance of particular taxa (Grunewald & Schubert, 2007), and can be calculated by Equation 1:

$$H = -\sum_{i=1}^{n} p_i \, ln \, p_i$$

Equation 1: Shannon diversity [H] (Shannon, 1948). In a community of n species the proportion p_i of the total individuals belongs to species i (Colwell, 2009).

Faith's PD is calculated by adding together the branch lengths for the present taxa in a sample to estimate the total phylogenetic diversity. Faith's PD does not account for ASV abundance but instead uses only presence or absence of information (Faith, 1992).

I performed three GLMMs to predict observed richness, Shannon diversity, and Faith's PD. Each model was created with a Gaussian distribution and meerkat IDs as a random effect. TB status (PCR) and TB symptom status were included as fixed variables, and sequencing depth, storage methods, physical condition, meerkat sex, age at sampling, and days until death were also included in the model to control for any effects they may have. These global models, where all variables are included, were presented in order to compare effects of all variables across models. The optimum models were chosen by Akaike information criterion (AIC), using the "MuMIn" R package (Bartón, 2020), and effects of variables retained in the optimum models were visualized.

2.5.2 Beta diversity

Beta Diversity represents the inter-community or between individual diversity, and signifies how different one community is from all other communities in the sampled population (Anderson et al., 2006; Thukral, 2017). The Bray-Curtis dissimilarity puts the number of species unique to any of both observed sites in relation to the sum of abundances in both sites. It therefore determines the dissimilarity of communities, takes values between 0 (similar) and 1 (dissimilar), and can be calculated according to Equation 2. By being equally effected by balanced abundance variation and abundance gradients the Bray-Curtis dissimilarity is useful for evaluating biodiversity patterns and their causes (Baselga, 2013).

$$d_{BC} = \sum_{i} \frac{|x_{ij} - x_{ik}|}{(x_{ij} + x_{ik})}$$

Equation 2: Bray-Curtis index of dissimilarity. The abundance of species i on site j is x_{ij}, the abundance of species i on site k is x_{jk} (Baselga, 2013).

The UniFrac metric is based on the measurement of the fraction of the branch length of a phylogenetic tree to determine the phylogenetic distance between two taxa (Lozupone & Knight, 2005). While unweighted UniFrac is a qualitative measure of beta diversity, using the presence or absence of taxa to compare community composition, weighted UniFrac is a quantitative measure, additionally considering the relative abundance to detect community changes (Lozupone et al., 2007). The Bray-Curtis index, unweighted UniFrac, and weighted UniFrac were calculated utilizing the "phyloseq" R package (McMurdie & Holmes, 2013).

Statistical testing was performed by creating three permutational multivariate analysis of variance (PERMANOVA) models to predict Bray-Curtis dissimilarity, unweighted UniFrac, and weighted UniFrac by using the "adonis" function of the "vegan" R package (Oksanen et al., 2012).

TB status (PCR) and TB symptom status were included as fixed variables, and sequencing depth, storage methods, physical condition, meerkat sex, age at sampling, and days until death were also included to control for any effects they may have on beta diversity. The three PERMANOVA models were calculated in order to analyze the effects of these fixed variables on beta diversity. Visualization was accomplished with the ordination method of principle coordinates analysis (PCoA).

3 RESULTS

3.1 TB INFECTION DISTRIBUTION AND EFFECT ON PHYSICAL CONDITION

I analyzed 362 samples from 58 individuals to investigate the effects of a *M. suricattae* infection on the gut microbiome of wild meerkats. 203 samples were PCR negative, and 159 samples were PCR positive, denoting a TB infection. 18 individuals remained PCR negative throughout their life, while 40 meerkats were infected eventually. A sample timeline matching samples to individuals and indicating the PCR results is shown in Figure 3.

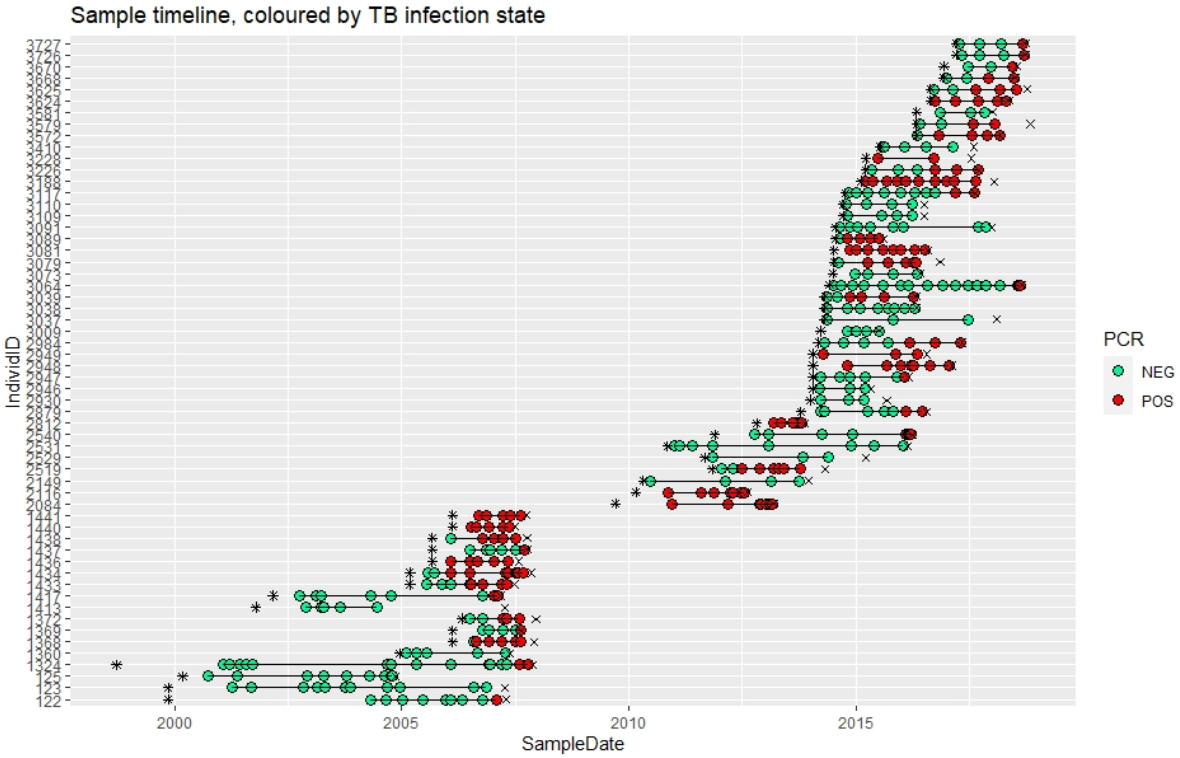

Figure 3: Sample timeline for 362 samples from 58 individuals. Negative PCR results are green, positive PCR results are red and indicate a *M. suricattae* infection based on PCR. Birth dates are marked with asterisks and crosses denote death date.

Hypothesis I: Uninfected meerkats are in better physical condition than infected meerkats

Uninfected meerkats were in better condition than TB infected individuals, although variation in both groups existed (Figure 4). According to this a TB infection is conveying statistically significant negative effects on meerkats, associated with a lower physical condition (Z = -2.885, p = 0.004).

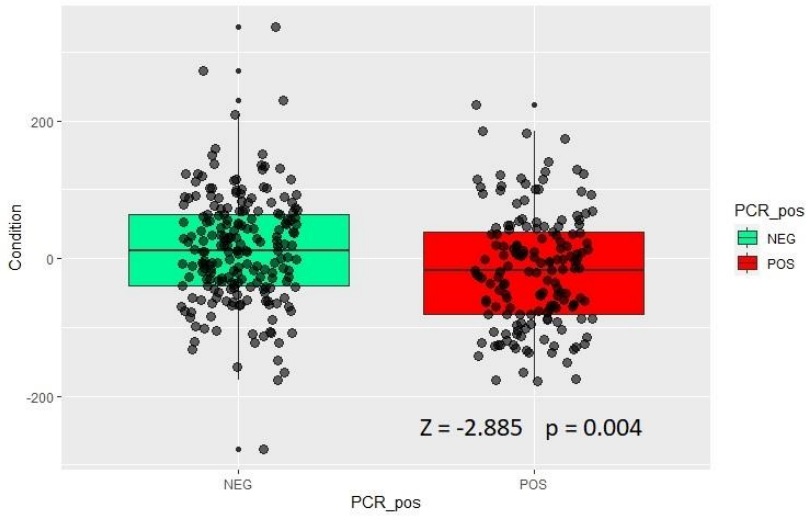

Figure 4: Boxplot illustrating the statistically significant negative effects of a *M. suricattae* infection on the physical condition of meerkats (Z = -2.885, p = 0.004). Negative PCR results (green) represent uninfected samples, whereas positive PCR results (red) indicate infection.

3.2 DESCRIPTION OF GUT MICROBIOTA COMPOSITION ACROSS THE POPULATION

The 362 samples in the final dataset resulted in a total of 9,486,576 sequences with a mean sequencing depth of 26,206 reads. The maximum was 79,161 reads per sample and the minimum was 8,431 reads per sample. Normalization of the 362 samples by rarefaction led to a total of 2,896,000 reads being analyzed hereinafter. These are including 11,474 ASVs.

Firmicutes showed with 75.25 % the highest relative abundance at the taxonomic rank phylum (Figure 5). The second most abundant phylum were Actinobacteria with 10.14 %. The family Clostridiaceae (Firmicutes) was present with the highest prevalence and an abundance of 23.36 %, followed by Lachnospiraceae (Firmicutes) with an abundance of 17.96 %. The relative abundance at family level across samples and individuals is visualized in Figure 6.

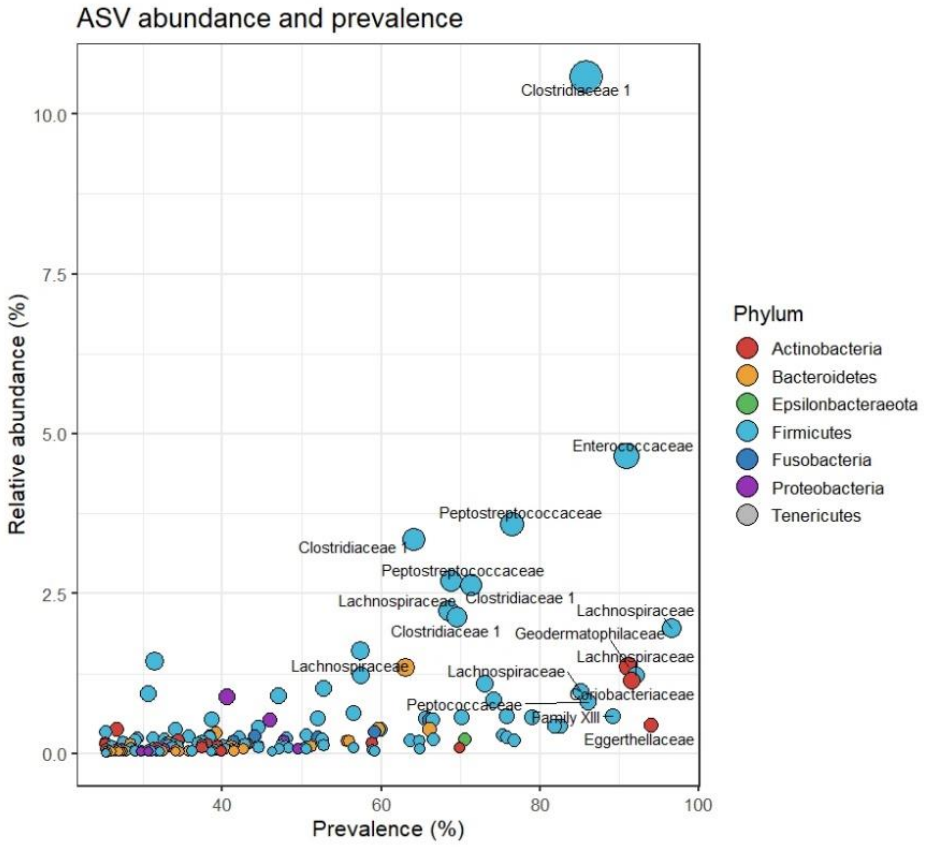

Figure 5: Relative abundance and prevalence of ASVs in the samples on family level. ASVs are colored by their phylum, the circle size represents relative abundance.

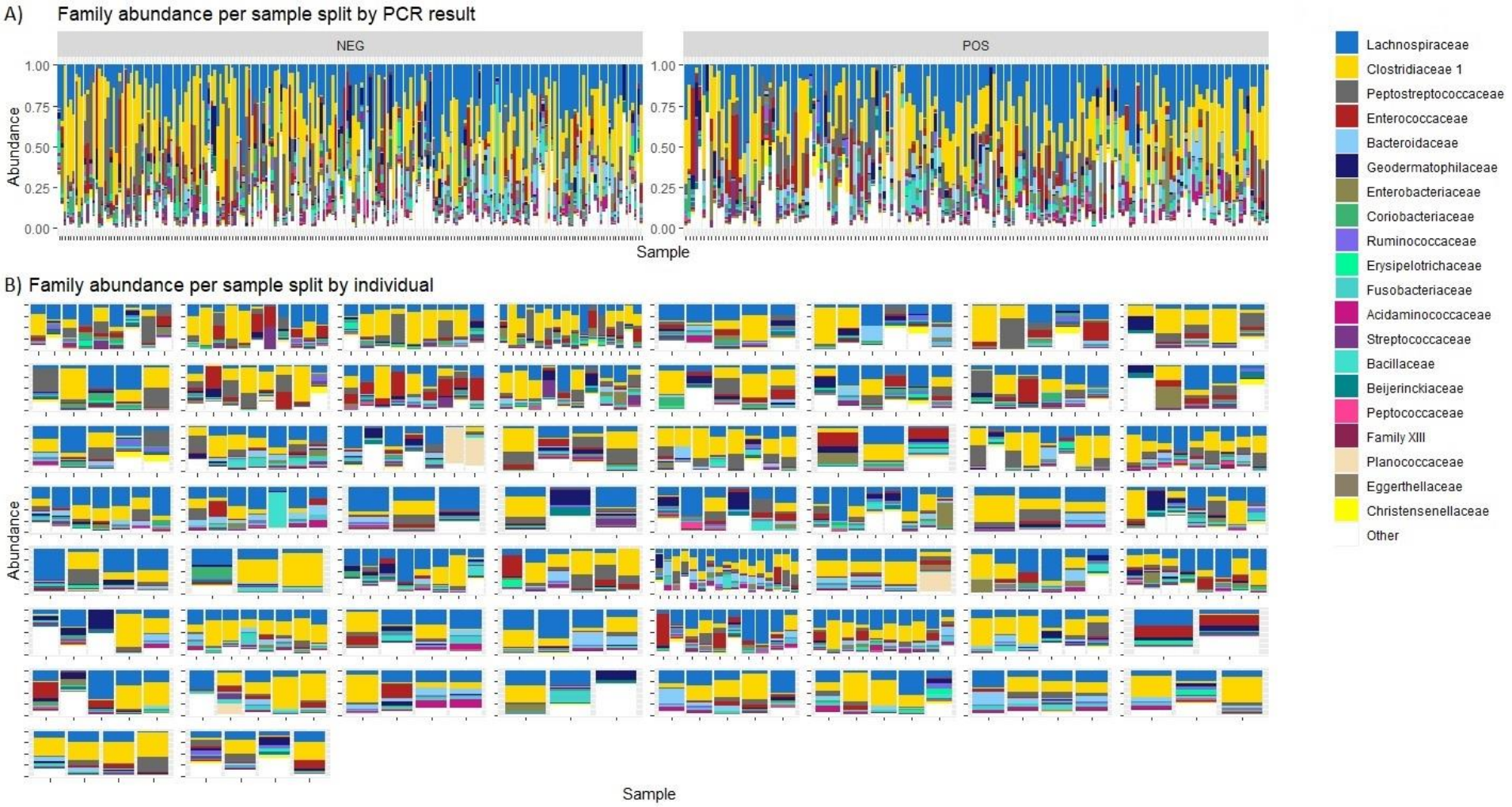

Figure 6: Barplots of ASVs per sample on family level. A) Comparison of family abundance between PCR positive and PCR negative samples. B) Illustration of family abundance, split by individual. The legend is showing the 20 most abundant families, all remaining families are merged as “Other”.

3.3 EFFECT OF TB ON ALPHA DIVERSITY

Hypothesis IIa: Variation in alpha diversity is higher in infected versus uninfected samples if effects of TB infection are stochastic

I predicted that TB infection would increase variation in alpha diversity if TB infection destabilized the gut microbiome. I found that contrary to this prediction there was no difference in alpha diversity between uninfected and infected samples, nor in variation in alpha diversity (Figure 7A; Table 1). None of the alpha diversity indices tested showed any statistically significant difference between TB uninfected and infected meerkats. The composition of the microbiome is therefore not linked to *M. suricattae* infection of the host and this hypothesis can be rejected.

Hypothesis IIb: Because latent infections often do not affect host health, samples taken from TB symptomatic meerkats may therefore vary more in alpha diversity than those from non-symptomatic individuals

Because meerkats can be infected with TB while not showing any symptoms, I also tested whether TB symptom status was associated with alpha diversity, since active symptoms indicate that *M. suricattae* is replicating extensively in the lungs and the meerkat is infectious (Marimani et al., 2018). However, I found that the median values of non-symptomatic and symptomatic individuals did not differ for observed richness, Shannon diversity and Faith's PD (Figure 7B; Table 1). This means that hypothesis IIb can be rejected as well, suggesting that the gut microbiome does not differ between non-symptomatic and symptomatic meerkats.

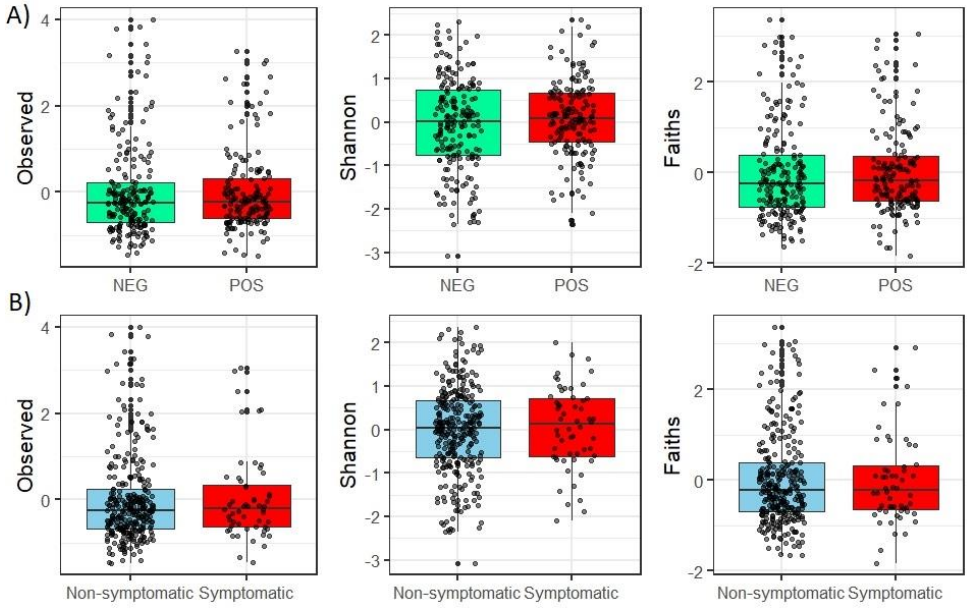

Figure 7: Boxplots showing effects of A) *M. suricattae* infection and B) symptoms on ASV observed richness, Shannon diversity and Faith's PD.

Table 1: GLMMs, containing the meerkats ID as random effect, applied to test the significance of the effects of all variables within the dataset on A) ASV observed richness, B) Shannon diversity, and C) Faith's PD. R^2 is 0.34 for A), 0.31 for B), and 0.32 for C).

A) ASV observed richness	Estimate	Std. Error	z value	P value
(Intercept)	0.139	0.108	1.290	0.197
TB status (PCR)	-0.081	0.118	-0.689	0.491
TB symptom status	-0.018	0.153	-0.116	0.908
Sequencing depth	**0.310**	**0.050**	**6.176**	**<0.0001**
Storage method	0.016	0.120	0.138	0.891
Physical condition	0.014	0.052	0.268	0.789
Sex	-0.151	0.108	-1.398	0.162
Age at sampling	-0.096	0.053	-1.799	0.072
Days until death	**-0.145**	**0.058**	**-2.509**	**0.012**
B) Shannon diversity	Estimate	Std. Error	z value	P value
(Intercept)	0.214	0.115	1.865	0.062
TB status (PCR)	-0.031	0.123	-0.248	0.805
TB symptom status	-0.129	0.158	-0.812	0.417
Sequencing depth	-0.023	0.052	-0.441	0.660
Storage method	**-0.264**	**0.128**	**-2.064**	**0.039**
Physical condition	0.048	0.056	0.870	0.385
Sex	-0.164	0.114	-1.431	0.153
Age at sampling	-0.094	0.058	-1.629	0.103
Days until death	**-0.241**	**0.060**	**-3.992**	**<0.0001**
C) Faith's PD	Estimate	Std. Error	z value	P value
(Intercept)	0.148	0.109	1.360	0.174
TB status (PCR)	-0.025	0.119	-0.213	0.831
TB symptom status	-0.087	0.155	-0.561	0.575
Sequencing depth	**0.272**	**0.051**	**5.369**	**<0.0001**
Storage method	-0.026	0.121	-0.215	0.829
Physical condition	-0.006	0.053	-0.115	0.909
Sex	-0.168	0.109	-1,537	0.124
Age at sampling	-0.094	0.054	-1.748	0.081
Days until death	**-0.139**	**0.058**	**-2.387**	**0.017**

Other factors influencing alpha diversity

The only three variables within the dataset that showed a statistically significant effect on alpha diversity were sequencing depth, storage method, and days until death (Figure 8). Storage method and days until death were statistically significant following the Shannon index (Figure 8B), whilst sequencing depth and days until death were associated with observed richness and Faith's PD (Figure 8A and C). The condition of meerkats, their sex, and their age at sampling weren't predicted to influence alpha diversity of the meerkats' gut microbiome significantly by any index.

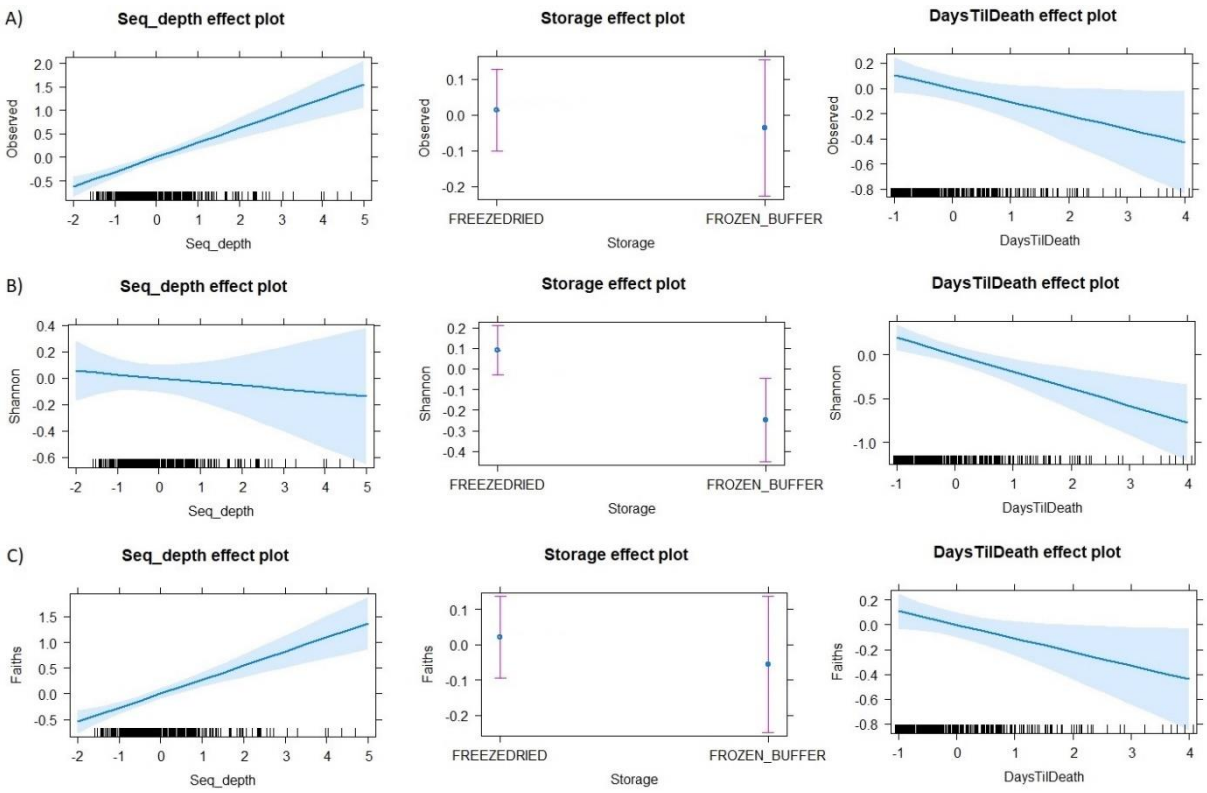

Figure 8: Effect plots visualizing the statistically significant effects of the variable days until death following A) ASV observed richness, B) Shannon diversity, and C) Faith's PD. Additionally sequencing depth was significantly associated with ASV observed richness and Faith's PD, while storage method (error bars represent standard errors) was significant following Shannon diversity. Optimum models were chosen by implementation of AIC.

3.4 EFFECT OF TB ON BETA DIVERSITY

Hypothesis IIIa: Dispersion in beta diversity is higher when meerkats are infected versus uninfected, according to the Anna Karenina principle

I predicted that if TB infection causes community-wide changes to the gut microbiome, this would generate differences in beta diversity between uninfected and infected samples. However, there was no difference in beta diversity between these groups when measured by Bray-Curtis dissimilarity (Figure 9A), unweighted UniFrac dissimilarity (Figure 9B), and weighted UniFrac dissimilarity (Figure 9C). This observation can be confirmed statistically when looking at the PERMANOVA models of these indices in Table 2, confirming no significant difference between the gut microbiome of uninfected and infected meerkats (Bray: $F = 0.735$, $p = 0.825$; Unweighted UniFrac: $F = 0.406$, $p = 0.991$; Weighted UniFrac: $F = 0.459$, $p = 0.868$). In addition, there was no difference in dispersion between uninfected and infected samples (Bray: $F = 0.260$, $p = 0.608$; Unweighted UniFrac: $F = 2.120$, $p = 0.149$; Weighted UniFrac: $F = 0.129$, $p = 0.718$).

Hypothesis IIIb: Samples taken when an individual is non-symptomatic may be more similar in their beta diversity to uninfected than infected states, since latent infections are often undetectable and with little effect on host health

Although health of individuals is only slightly affected by latent TB infection, it often acutely decreases in late infection stages (Drewe et al., 2009). However, beta diversity between non-symptomatic and symptomatic meerkats did not differ according to Bray-Curtis dissimilarity (Figure 9D), unweighted UniFrac dissimilarity (Figure 9E), and weighted UniFrac dissimilarity (Figure 9F) measurements, as well as PERMANOVA models (Table 2: Bray: $F = 1.204$, $p = 0.210$; Unweighted UniFrac: $F = 0.369$, $p = 1.012$; Weighted UniFrac: $F = 0.687$, $p = 0.661$), and thus this hypothesis can be rejected as well. Furthermore, there was a difference in dispersion between samples from non-symptomatic and symptomatic meerkats following unweighted UniFrac dissimilarity, but dispersion was similar when measured by Bray-Curtis dissimilarity and weighted UniFrac dissimilarity (Bray: $F = 0.732$, $p = 0.394$; Unweighted UniFrac: $F = 4.647$, $p = 0.030$; Weighted UniFrac: $F = 1.342$, $p = 0.243$).

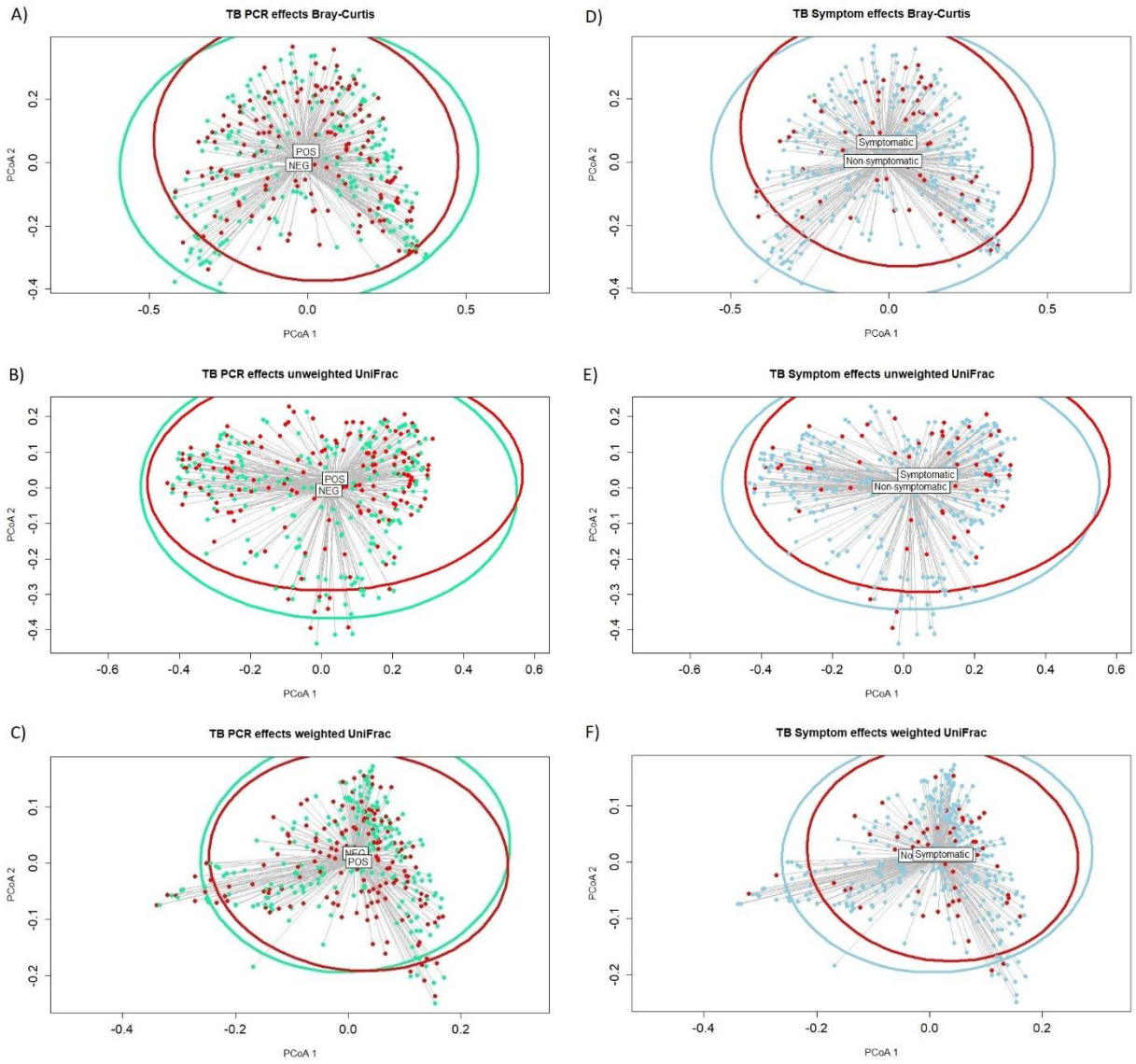

Figure 9: PCoA plots showing differences in beta diversity for A) TB infection effects based on Bray-Curtis dissimilarity, B) TB infection effects based on unweighted UniFrac, C) TB infection effects based on weighted UniFrac, D) symptom effects based on Bray-Curtis dissimilarity, E) symptom effects based on unweighted UniFrac, and F) symptom effects based on weighted UniFrac. Centroids are green for samples from uninfected meerkats, blue for samples from non-symptomatic individuals, and red for samples from TB infected as well as symptomatic meerkats.

Other factors influencing beta diversity

Whilst neither TB infection nor TB symptom status were associated with beta diversity, sequencing depth, storage method, the age of the meerkat at sampling, as well as proximity to death, appeared to have small effects ($R^2 \leq 0.021$) on all three measures of beta diversity (Table 2). Physical condition at the time of sampling also appeared to have a small effect on beta diversity when measured by Bray-Curtis and unweighted UniFrac. However, again this effect is very small ($R^2 \leq 0.006$). There was no effect of sex on beta diversity for either measure. Overall, whilst some factors appeared to differ significantly in their beta diversity centroids, I found no factor that strongly explained variation in beta diversity.

Table 2: PERMANOVA models to test significance of the effects of all variables within the dataset on A) Bray-Curtis dissimilarity, B) unweighted UniFrac, and C) weighted UniFrac.

A) Bray-Curtis dissimilarity	Df	SumsOfSqs	MeanSqs	F. Model	R^2	P value
TB status (PCR)	1	0.233	0.233	0.735	0.002	0.825
TB symptom status	1	0.382	0.382	1.204	0.003	0.210
Sequencing depth	**1**	**0.732**	**0.732**	**2.309**	**0.006**	**0.005**
Storage method	**1**	**2.474**	**2.474**	**7.806**	**0.021**	**0.001**
Physical condition	**1**	**0.725**	**0.725**	**2.287**	**0.006**	**0.001**
Sex	1	0.466	0.466	1.469	0.004	0.063
Age at sampling	**1**	**1.011**	**1.011**	**3.191**	**0.009**	**0.002**
Days until death	**1**	**0.843**	**0.843**	**2.660**	**0.007**	**0.001**
C) Unweighted UniFrac	Df	SumsOfSqs	MeanSqs	F. Model	R^2	P value
TB status (PCR)	1	0.230	0.230	0.991	0.003	0.406
TB symptom status	1	0.235	0.235	1.012	0.003	0.369
Sequencing depth	**1**	**1.074**	**1.074**	**4.616**	**0.012**	**0.001**
Storage method	**1**	**1.260**	**1.260**	**5.419**	**0.015**	**0.001**
Physical condition	**1**	**0.405**	**0.405**	**1.742**	**0.005**	**0.040**
Sex	1	0.310	0.310	1.331	0.004	0.121
Age at sampling	**1**	**0.417**	**0.417**	**1.792**	**0.005**	**0.030**
Days until death	**1**	**0.624**	**0.624**	**2.685**	**0.007**	**0.003**
C) Weighted UniFrac	Df	SumsOfSqs	MeanSqs	F. Model	R^2	P value
TB status (PCR)	1	0.016	0.016	0.459	0.001	0.868
TB symptom status	1	0.024	0.024	0.687	0.002	0.661
Sequencing depth	**1**	**0.077**	**0.077**	**2.207**	**0.006**	**0.041**
Storage method	**1**	**0.274**	**0.274**	**7.876**	**0.021**	**0.001**
Physical condition	1	0.042	0.042	1.218	0.003	0.274
Sex	1	0.055	0.055	1.575	0.004	0.142
Age at sampling	**1**	**0.083**	**0.083**	**2.382**	**0.006**	**0.025**
Days until death	**1**	**0.097**	**0.097**	**2.772**	**0.007**	**0.015**

4 DISCUSSION

In this study, I investigated the effects of TB infection on gut microbiome composition of wild meerkats by amplification of a fragment of the 16S rRNA gene, application of the Illumina high throughput sequencing method to create a DNA Amplicon sequencing library, and subsequent statistical analysis with bioinformatic tools. The meerkats' gut microbiome was dominated by the phylum Firmicutes, showing a relative abundance of 75.25 %, followed by Actinobacteria with 10.14 %. On family level Clostridiaceae (23.36 %, Firmicutes) and Lachnospiraceae (17.69 %, Firmicutes) were the most abundant. Against expectations I could not detect significant alterations in alpha or beta diversity induced by *M. suricattae* infection. While studies in humans (Luo et al., 2017; Maji et al., 2018) and mice (Winglee et al., 2014) reported changes in composition and diversity of microbial communities due to pulmonary TB infection, as pointed out in the introduction, this study shows contradicting results. Furthermore, no stochastic changes in gut microbiome composition according to the Anna Karenina principle could be detected, as beta diversity did not increase and centroids in the PCoA plots (Figure 9) showed similar dispersion for TB uninfected compared to infected individuals.

The results obtained here demonstrate that TB infection has little effect on overall gut microbiome diversity in meerkats. These findings are in line with some previous research, also failing to find an association between TB infection and microbiome composition. For example, *M. tuberculosis* infected rhesus macaques had the same gut microbiome alpha diversity as uninfected individuals (Namasivayam et al., 2019). However, infection was associated with beta diversity in this study. These alterations were extremely small, however, and variation between individuals was much greater than that attributed to infection status (Namasivayam et al., 2019). Another study in mice observed an initial loss of alpha and beta diversity arising six days after infection, followed by recovery of gut microbial diversity to an alternative stable state (Winglee et al., 2014). In contrast, an initial increase in beta diversity of lung microbiota at one month after TB infection was reported in cynomolgus macaques (Cadena et al., 2018). Diversity returned to baseline after four months, but relative abundance of various taxa remained altered as well. Together, these studies suggest that in controlled settings small TB effects on the microbiome can be detected, yet these are likely to be overshadowed by natural variation and temporal fluctuations that characterize gut microbiomes of wild animals.

The major methodological difference of my study on meerkats to other studies that investigate the same topic is that mice and macaques were systematically infected with TB under very controlled conditions. As a result, information on the exact date of infection was available and sampling was carried out in short, regular time intervals for these studies. Since the exact date of TB transmission is not known in this study it is possible that short-term changes to gut microbiome structure immediately following infection occurred but went undetected here. However, this study aimed to detect long-term dysbiotic shifts in microbiome composition following infection, and results from this study clearly demonstrate no such community-wide shift occurs. Future studies on meerkats need to implement short, frequent sampling intervals additional to obtaining information on the date of infection in order to be able to detect potential short- and long-term microbiome diversity changes linked to TB infection.

Although no associations between TB infection and alpha and beta diversity were observed, I detected significant associations of TB infection with the physical condition of meerkats. Infected individuals exhibited lower weight than predicted for their age, indicating negative effects of TB infection on physical condition. This is in line with other studies that tested for this link (e.g. rhesus macaques (Namasivayam et al., 2019)). In rhesus macaques, disease severity and subsequent weight loss was identified as the variable most significantly associated with microbiome composition. Interestingly, distinct baseline microbiome compositions prior to infection could be identified for animals developing severe and less severe disease. The authors hypothesized that specific bacterial communities may either directly influence host susceptibility towards TB infection and disease severity, or that they represent indirect biomarkers of other host differences that are affecting pathogenesis (Namasivayam et al., 2019). Control samples of meerkats that never were exposed to TB must be integrated in future studies to potentially identify such distinct baseline microbiome compositions in meerkats, as most of the individuals sampled for this study were exposed to TB from birth. Since development of symptoms in meerkats with active TB infection indicates that *M. suricattae* is replicating extensively in the lungs and that these individuals can transmit the pathogen (Marimani et al., 2018), I tested for associations of TB symptom status and microbial diversity. Against my assumption that samples taken from TB symptomatic meerkats show greater variation in alpha and beta diversity, since latent infections often do not affect host health, no associations could be found. However, I did not test for effects of TB on specific lineages, and it is a possibility that TB infection may alter abundance of specific taxa. If these taxa are of functional importance, then the alterations of a small number of taxa may be connected with the decrease in host physical condition after TB infection.

I detected statistically significant influence on gut microbiome diversity of several methodological (sequencing depth, storage method) and biological (physical condition, age at sampling, proximity to death) variables. These differences appeared to arise from a large sample size that leads to very close, yet not overlapping centroids in the PCoA plots, suggesting such small effects may not be ecologically meaningful, because these parameters explained only between 0.5 to 2.1 % of variation in microbiome beta diversity. Therefore, sequencing depth and storage method did not bias the results. However, no biological factors could be identified to influence microbiome dynamics. While it is assumed that human microbiome composition varies depending on age (O'Toole, 2012), and another study identified gender as a factor affecting homeostatic microbiome stability (Gao et al., 2018), no such associations could be detected in meerkats.

In conclusion, this study provided an initial insight into the response of gut microbiome composition in meerkats to *M. suricattae* infection. While the presented results did not match my expectations of TB induced alterations in alpha and beta diversity of microbial communities, the study highlights the influence the host microbiome may exert on susceptibility towards and outcome of disease. Nevertheless, this study does not rule out the possibility of TB infection affected specific bacterial taxa. Experimental research, extensively sampling uninfected and infected humans and mammals in short time intervals before, during, and after TB infection under as controlled conditions as possible, are required to unveil mechanisms affecting multidirectional interactions between microbiome composition and various host factors, and to obtain a better understanding for drivers of microbiome dynamics associated with TB infection.

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