A CASE CONTROL STUDY OF CHRONIC INTERMITTING DIARRHEA IN MACACA NIGRA HELD IN CAPTIVITY

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Abstract

Background:

Chronic diarrheal disease (CDD) is a serious issue amongst macaques and nonhuman primates (NHP) in captivity. A group of Celebes Crested Macaques (*M.nigra*) suffers from CDD symptoms since 2015. Despite several diagnostic and therapeutic efforts made by the Zoo, the animals have not improved.

Objective

The general aim is 1) to investigate the causation of the chronic intermitting diarrhea in *M.nigra* at the Zoo, and 2) to determine the possible multifactorial influences (considering the diet, type of housing, possible pathogens, and the composition of the microbiome). This research focusses on establishing the most effective fecal identification method for this particular group of *M.nigra.* The second focus of this study will be to examine the digestion capacity and give an insight in the microbiome of this study group, considering that the cause of CDD is multifactorial.

Study Design

This prospective, observational case-control study. This study examines the fecal identification methods applicable to the study group (*N*=4). Furthermore, the digestion rates of the study group and control groups of *M.mulatta* ('healthy' and 'with diarrheal symptoms') are examined and compared. Finally, an insight in the microbiome of the study group will be given.

Population

The study group (*N*=4) of *M.nigra* consist of two adult females and two juvenile females. The control groups consist of healthy *M.mulatta* and individuals with diarrheal symptoms. The control groups are kept in 5 different enclosures at the 'Biomedical Primate Research Centre' (BPRC).

Methods

The most accurate fecal identification method is examined by comparing beads with consumable glitters and non-consumable-nontoxic glitters. The color retention, the color reflections, the color combinations, and the applicability is examined.
A fecal examination protocol and a sampling protocol is designed. Samples of the study group are compared to the control groups on macroscopic, microscopic features, and their digestive enzymes. Fecal samples are sent to the 'BaseClear' facility for the profiling and determination of the microbiomes of this study group, using Next-Generation Sequencing (NGS) technology.
Statistical analyses are made using the SPSS program perform the Wilcoxon paired T-test, Mann-Whitney U-test, and ANOVA-test

Results

Most accurate fecal identification method for this study group is ½ tsp. of non-consumable-nontoxic 'Rainbow Dust' glitters in the colors 'Lime Green', 'Oasis Blue', 'Cherry Red', and 'Bubblegum'.
The pH was significantly higher in the samples with a Waltham-score of 4.0 or higher, and individual No.1 seemed to show the CDD symptoms most often. The number of fat in the fecal samples from the *M.nigra* population was substantially lower than the number of fat in the fecal samples from the *M.mulatta* population. No differences in fecal amylase activity were found. The fecal protease activity was significantly lower in May 2018 in the *M.nigra* group compared to the *M.nigra* group. However, this difference did not reoccur in June 2018. The main groups of bacterial phylae found in the study group the Firmicutes and the Bacteroidetes. Furthermore, no infectious pathogens were identified.

Conclusions

The most accurate fecal markers for this study group have been determined. And an overview and insight have been found of the digestion rates and microbiome of the study group, and the possible influences thereon. This study has built a foundation for the following studies examining the same main goal. In conclusion, a larger study group, and control group is needed to generate reference values of the microbiome and digestion rates of the study group

Abbreviations:

BCS	Body Condition Score
BPRC	'Biomedical Primate Research Centre'
CDD	Chronic diarrheal disease
EEP	European Endangered Species Breeding Program
ICD	Idiopathic chronic diarrhea
ITS	Internal transcribed spacer
IUCN	International Union for Conservation of Nature
PBS	Phosphate-buffered Saline
PCR	Polymerase Chain Reaction
SAF	Acetate-acetic Acid-formalin
SIV	Simian immunodeficiency virus
NGS	Next-Generation Sequencing
NHP	nonhuman primates
UKG	University Clinic of Utrecht
UVDL	'Universitair Veterinair Diagnostisch Laboratorium'

Contents

Introduction

The island of Sulawesi, Indonesia, is the endemic home of seven species of macaques, the Sulawesi crested black macaques (*Macaca nigra)* being one of them. The *M.nigra*, also known as the '*Macaca lembicus', 'Macaca malayanus', '*Celebes Crested Macaque', the 'Crested black macaque', and the 'black ape', is of the Cercopithecidae family. Four of the macaques are classified as vulnerable, one is endangered and the *M.nigra* is classified as a critically endangered species on the 'Red List' of the 'International Union for Conservation of Nature' (IUCN)[1]. It is also one of the top 25 most threatened primates[2]. In 2008 the estimated population density was approximately three individuals/km2, an introduced population of the Bacan island not included. According to Melfi et al. (2010) and Palacios et al. (2011) the largest remaining population, in the Tangkoko Reserve, consists of less than 2.000 individuals[3,4]. The crested Macaques are hunted by the local people for bushmeat. Flourishing agriculture is one of the causes for a loss of lowland forest habitat, resulting in fragmentation of the habitats of the subpopulations living in Sulawi[5]. The small size of the subpopulations raises concerns about the genetic pool of these subpopulations. A research on genetic markers useful for studies on evolutionary biology of crested macaques, indicates that the subpopulation in Tangkoko still seems genetically variable. From which arises the hope that this might also still be the case in other subpopulations[6]. Their subpopulations consist mostly of multi-male multi-female groups with an alpha male at the top of the hierarchy[7].

In nature, the *M.nigra* spends 59% of its time moving and searching for and consuming food and 21.8% of its time in social activities. The other 19.5% of its time is spent on resting. They are predominantly frugivorous. Meaning, the 59% spent on moving and consuming food, was mostly spend on the consumption of fruits. Their documented consumption time was spent consuming over 145 fruit species from over 36 families. Most fruits were digested ripe, some fruit and some unripe seeds. The rest of their diet consisted of invertebrates, unripe seeds, leaves, flowers, pith, terminal shoots, herbs, grass, seeds, and fungus. A few times they were seen eating eggs, a forest gecko, a frog or even an adult fruit bat. Small juveniles ate fruits significantly more often than adult males. And the smaller sized groups also ate more fruit than the large group. The average daily path length was 2388m per day of the month[8].

The *M.nigra* is being held as a pet and is predominantly held in European Zoos. The 'European Endangered Species Breeding Programme' (EEP) is managing the genetic stock as an insurance population with potential for restocking. Captivating wildlife can come with several challenges, diarrhea being one of them. Diarrhea has been a long-lasting problem in captive macaques. It is a significant source of morbidity and mortality[9]. Based on knowledge of diarrhea in monkeys as well as other mammals, this diarrhea can have multiple causes, including infectious and non-infectious causes, or potentially result from a combination of the two. When multiple animals are affected, the most likely causes appear to be related to either specific pathogens, alterations of the gut microbiome (so-called dysbacteriosis), dietary/nutrition-related problems, stress and housing/management related issues, usage of antibiotics, and/or ingestion of toxins (e.g., zinc toxicity). Research of Kanthaswamy et. al (2014) suggests that chronic diarrheal disease (CDD) in Rhesus Macaques kept in captivity is under strong genetic control[10]. Similarly, idiopathic chronic diarrhea (ICD) has been identified as a frequent cause of morbidity and mortality among juvenile rhesus macaques. ICD causes histopathologic changes, while macroscopic lesions may be absent[11]. Other studies have shown a potential relationship with housing, age and/or seasonp[9]. Of the various infectious agents that can occur, simian immunodeficiency virus (SIV), adenoviruses, rotaviruses, primate caliciviruses, *Campylobacteraceae, Shigella, Yersinia enteroclitica, Salmonella, Clostridium difficile, Helicobacter spp., Bacteroidetes, Firmicutes* phyla, *Strongyloides fulleborni, Balantidium coli, Giardia lamblia,* and *Cryptospodidium,* are mentioned the most in research on diarrhea causes in

Macaques. However, multiple of these viruses, bacteriae, and parasites have also been found in animals without diarrhea[9,12–16]. This implicates that finding one of these infectious agents does not necessarily implicate that this is the definite cause of the symptoms. However, research suggests that the diversity of the microbiome plays an important role in the protective role in response to enteric pathogens. The microbiome also influences the immunity of the host, and contributes/influences the digestion capacity of the host[16–20]. The cynomolgus macaques originated from the Philippines had a lower diversity in their microbiome compared to those originated from Mauritius and was more susceptible to the wildtype S.*dysenteriae* than those originated from the Philippines[20]. This study suggests that a more diverse intestinal microbiota may play a protective role in response to enteric pathogens. Stress is an important factor that can influence an individual's immune system and thereby, influence the susceptibility of the animal to infectious diseases. Different studies have also shown that stress can be the cause of a reduction in gastrointestinal microbial diversity[20]. Visitors of the Zoo, mixed species interactions, and the possibility of foraging are just a few examples of the many of behaviors that influence the well-being and stress levels of these animals[21].

This research is based on a case of chronic intermittent diarrhea, which has been observed in the group of crested macaques (*M.nigra*) at a Zoo in the Netherlands. The group, which currently consists of four females of different ages has had this problem since 2015. As a result of the diarrhea, three out of four animals appear to have a low body condition score (BCS). In 2017 two other Celebes Crested Macaques of the group had died. One, a male that died of purulent pleuropneumonia. The other, a three-year-old female died of a *Yers. Pseudotuberculosis* infection and an adult male of a purulent pleuropneumonia. The *Yersinia pseudotuberculosis* was confirmed with Polymerase Chain Reaction (PCR). The feces of the other individuals were tested for *Yersinia Pseudotuberculosis* infection and were tested negatively with PCR. Histologic examination of the intestines of the young female showed shallow intestinal villi. After the passing of the two individuals, fecal examinations have been performed to evaluate the presence of specific bacterial, fungal, and parasitic pathogens. None were found in the feces of the remaining four individuals, apart from many flagellates in one sample. Interestingly, after treatment with Panacur® (*Fenbendazole*) and *Metronidazole*, both treatments resulted in worsening the clinical signs of diarrhea. Leading to the conclusion that the flagellates most likely were commensals. Since the start of the diarrhea problems, their diet has been evaluated and recalculated many times, without any long-term improvements to the issues. The diarrhea problems are at their worst during the spring and the fall but remain visible during the entire year.

The lack of improvements to the diarrhea problems even after dietary adjustments, fecal examinations and medical therapy indicates that this is a complex and possibly multifactorial problem. Due to the chronicity of the problem, the Zoo has consulted specialists at the University Clinic of Utrecht (UKG) to collaborate in finding the cause for the diarrhea in the animals.

Aim of the study

The aim of this research is 1) to investigate the causation of the chronic intermitting diarrhea in *M.nigra* at the Zoo and 2) to determine the possible multifactorial influences (considering the diet, type of housing, possible pathogens, and the composition of the microbiome). This goal is to be accomplished by comparing the group of *M.nigra* at the Zoo with multiple groups of *Macaca mulatta* kept in another institution in the Netherlands.
The research goal will be accomplished by the following hypothesis:

H_o = There is no correlation between the diarrheal symptoms on the one hand and presence and ratio of certain micro-organisms, dietary or environmental factors on the other hand.

H_1 = There is a correlation between the diarrheal symptoms on the one hand and the presence and ratio of certain micro-organisms, dietary or environmental factors on the other hand.

Before these hypotheses can be answered the following uncertainties must be clarified:

1. How to distinguish the fecal samples from the individuals of the group?
2. What are the proportions in the average diet of the study group of *M.nigra* held in captivity and are these compatible with their dietary requirements in the wild?
3. What is the digestion capacity of the 4 individuals compared to a similar group of individuals held in another facility?
4. What is the microbiome of the 4 individuals, and is there a significant difference between them that correlates with the symptoms of diarrhea?

Materials and methods

Animals and Study design

Study Group

The subjects of this study were 4 Celebes Crested Macaques (*M.nigra*) in a social group at a Zoo in the Netherlands. The group consisted of 4 females at the time of this study, with the ages of 19, 14, 2, and 1.5 years old. The adult females have an estimated BCS of 3 and 1.5 out of 5[22,23]. Because this estimation has been made from a distance by the caretakers of the Zoo, the BCS could possibly be even lower. The records of the Zoo show signs of intermitting diarrhea since 2009. The group roams freely between an indoor residence and an outdoor residence, connected through tunnels. The tunnels to the outdoor residence are cleaned every 3 Months. The indoor residence has one large viewing window, multiple large rocks, and multiple tree trunks. The outdoor residence is surrounded by water. The outdoor residence consists of an island containing a lot of natural bushes, trees, large rocks, and grass. Giving the Celebes Crested Macaques a lot of hidings spots from the visitors. Visitors can view the Celebes Crested Macaques from a distance by walking on bridges on the other side of the water. Every morning at 8.00 am, the group is fed primate pellet and rice (for marking or medicine), after which the animals will be locked out of the indoor residence until 10.00 am. In which the indoor enclosure will be thoroughly cleaned. After 10.00 am the animals can roam freely between both residences the whole day. In the afternoon 1300 grams of vegetables (tomatoes, cucumber, fennel, daikon radish, Bell pepper, beans, broccoli, eggplant, zucchini, and celery) and 400 grams of starch vegetables (beetroot, pumpkin, corn, carrot, sweet potato, parsnip, kohlrabi, celeriac) will be thrown on the outdoor island. In the evenings, 1200 grams of leafy vegetables (endive, lettuce, watercress, spinach, parsley) will be scattered throughout the indoor residence. After which the animals usually prefer to stay indoors. Once a week they receive 10 boiled chicken eggs, and sometimes they also receive other animal proteins like crickets, mealworm, or grasshopper. Three times a week their enclosure will be enriched with mainly willow branches.

Control Groups

Control fecal samples were obtained from the 'Biomedical Primate Research Centre' (BPRC). The BPRC conducts research with java monkeys, white-eared brush monkeys and rhesus macaques (*Macaca mulatta)*. Several *M.mulatta* groups were used as control groups in this study. Fecal group samples and individual fecal samples were collected from 5 different groups of rhesus macaques kept at BPRC. The groups used for this study were not participating in any other study at the same time as the fecal sampling. Furthermore, the samples were obtained during the cleaning of the indoor residences and no individual food coloring was used, therefore this is not considered as an animal experiment. The indoor and outdoor resident of the rhesus macaques at BPRC contain Rocks, ropes, and tree trunks. No specific dietary information was collected from the control groups. The inclusion criteria for the control groups were that they should not be included in any other study that might cause conflict with our results. And that the groups participating in this study needed to consist out of rhesus macaques. Also, at least one of the groups needed to be free of diarrhea and one of the groups needed to have symptoms of diarrhea to the same extent as seen in the study group.

Study Design

This study is a prospective, observational study to evaluate whether the intermitting diarrheal symptoms concerned the whole group of Sulawesi crested black macaques or specific individuals, and whether infectious agents, differences in the gut microbiome, or dietary deficiencies, could be the cause of these symptoms. The aim for the future would be that microbiomes, diets and observational information would be shared between multiple Zoo's in Europe dealing with similar

problems. This study focusses on this specific group of animals, identifying their microbiomes, what type of diarrhea these animals experience and which individuals it includes. The correlation between stress and their microbiome and the influences of their behavior and diet on the individual's microbiome will be determined in another research. The welfare and care of study animals at study sites were ensured by overseeing staff members and veterinary supervision at the study site. Data was collected between the 1st of April 2018 and 27th of June 2018.
The structuring and planning of the study design resulted in several uncertainties that needed to be clarified before the primary aim of this study could be obtained. Resulting in the organization of multiple smaller studies within this study. First, the ideal fecal identification method needed to be determined to distinguish the samples of the four individuals living in group housing. This would enable the investigators to determine which individual(s) were showing signs of intermitting diarrhea and to what extent. Second, the fecal samples needed to be investigated macroscopically and microscopically to determine the digestion rates of the individuals. Third, the fecal sampling, -scoring and -conservation methods needed to be determined and reported into protocols. Most of the research and sampling have been done by the same pretrial trained observer or an official laboratory technician of an external facility. Whenever another observer participated, they were informed and trained by the protocols (*Attachment 1 of this study*), to minimalize the possibility of interobserver bias. Thirdly, external influences needed to be kept to a minimum and monitored. This was managed by keeping record of the diet of the study group, the weekly numbers of visitors, behavioral signs indicating stress or estradiol influences (scratching, aggression, redness and slapping on the buttocks) from the 8th of December 2017 until the 11th of June 2018. Dietary intake of the study group was regulated from the 6th of April until the 11th of June 2018.

Fecal identification methods

This part of the study includes determining the effectiveness of plastic beads, consumable glitters, and non-consumable-nontoxic glitters as markers to accurately distinguish the samples of the individuals. This is achieved by investigating the most effective color combinations, whether the markers absolve/loose color over time, the length of time between ingestion and detection in the feces, and the minimum quantity of marker needed for detection in the feces.
The study group were fed rice balls since the beginning of January 2018. Dietary and Waltham-scores were monitored throughout the diet change, no differences were reported in Waltham-scores by the zookeepers, indicating that the rice intake did not worsen the diarrheal symptoms ('*Waltham- Faeces Scoring System' Attachment 2 of this study*). Therefore, rice was used in this study to feed the markers to the study group.
Every morning at 8.00 am, the study group was given 200 grams of cooked white rice. After which the study group would be let outside to roam, while the indoor enclosure would be closed off for the animals and cleaned. This routine of the animals was kept as normal as possible by sampling the feces during the cleaning hours in the mornings. A 'fecal sampling form', and a 'fecal examination protocol' were designed to ensure that external factors were kept to a minimum. The 'fecal sampling form' explained how the fecal samples should be sampled, packed, labelled, and processed. The 'fecal examination protocol' explained which features of the fecal samples should be noted before and during the sampling of the feces and the examinations that needed to be done in the laboratory. Firstly, it needed to be determined what the gastrointestinal passage time would be of an individual with and without diarrhea. Therefore, the eldest adult (individual No. 1) was fed one rice ball with 'lime green' glitters on the first day of April 2018, followed with white glitters on the second day of April 2018. On the third day she was fed empty rice balls. The youngest adult (individual No. 2) was fed empty rice balls on the first and second day, and yellow glitters on the third day. The eldest of the juvenile females (individual No. 3) was fed nothing on the first day, lime green glitters on the

second day, and nothing on the third day. The youngest juvenile (individual No. 4) was fed nothing on the first day, white (and accidentally green) glitters on the second day, and Bubble-gum pink on the third day. After which all animals received empty rice balls for two whole days. When the samples were collected, it showed to be difficult to determine the identification of the fecal sample, due to the number of glitters and the different colors within samples. During this first trial, the possible influence of the non-consumable-nontoxic glitters on the gastrointestinal tract was questioned. Furthermore, with the current settings, the fecal samples could be 22 hours old when being collected. Causing a possible influence on pH and microbiome results. These concerns resulted in a secondary part of the study: Examining the pH of a single fecal sample preserved in 23 degrees Celsius (°C) for the seven following days. This temperature was chosen because it was the same temperature as the indoor enclosure of the study group at that time. Even though, no indications were found that the non-consumable-nontoxic glitters had a negative effect on the diarrheal symptoms, the comparison with consumable glitters and beads needed to be investigated further to minimize the risks of health hazards for the animals and to investigate the effectiveness before administering it long-term to the study group. To determine whether consumable glitters, non-consumable-nontoxic glitters, or beads needed to be used the following study was conducted: Fresh looking fecal samples (score: ≤2) were obtained from the enclosure and were scored on their digestion rate, their pH, their estimated age, and their consistency (following the Waltham fecal scoring system). The color of the feces and the possible contamination was also monitored. The Waltham fecal scoring system was used because all the Zookeepers were customed to use this scoring system. The colors of the consumable glitters used were: 'Purple', 'Red', 'Gold', 'Silver', 'Apple Green', and 'Ocean Blue'. The colors of the non-consumable-nontoxic glitters used were: 'Hologram white', 'Stardust Lime', 'Stardust Yellow', 'Stardust Bubblegum', and 'Yewel Ocean Spray'. The color of beads examined were white, pink, pearl, silver, green, purple, blue, and black. Of the glitters 0.1-0.2 grams of glitters were added to the fecal samples and 8 beads were added to each fecal sample. Over time it was monitored if the glitters and beads were still visible and if they would lose some or all their color, which would indicate a possible health hazard for the animal, and a less desirable identification marker. All the samples were kept at 37°C as a comparison with the temperature of the animal's gut. The samples were tested on the three following days and on day 30. After it indicated that the non-consumable-nontoxic glitters would be most effective in identifying the fecal samples to the individuals, the number of glitters and the specific color combinations needed to be determined. Research indicated that 0,25 tsp of glittering every 3 days was effective in DeBrazza's monkeys[24]. Even though unpublished, a research conducted by by A.Rox (2017, BPRC) of the BPRC compared food coloring with glitters (Attachment 3 of this study). This research concluded that the following combinations and dosage of glitters were effective in identifying fecal samples of *Macaca mulatta*: 'Red and Blue', 'Green and Bronze', 'Red and Bronze', 'Blue and Green', in a dosage of 2/3rd teaspoon of glitters, with a minimum of 1/2 teaspoon of glitters daily. Therefore, these colors (including the white and bubble-gum) were used to determine the most effective color-combination to identify fecal samples of four individuals. The process of administering the non-consumable-nontoxic glitters to the study group is described in the protocol 'Administering non-consumable-nontoxic glitters' (Attachment 4 of this study). To minimize the exposure, we again tested the fecal passage time by administering the first batch of glitters once and examined whether these colors were represented in the fecal samples in the following four days. We discovered that the time from ingestion to detection in the feces was less than 24 hours and up to 3 days. Therefore, we examined different color combinations every 5 days, so that we could be certain not to have any overlap in colors found in the feces. We also experimented with different dosages of glitters starting with 1/5th of a teaspoon and slowly heightening the dosage, resulting in half a teaspoon of glitters daily. To investigate the most effective color-combination for fecal identification, the following colors of non-

consumable-nontoxic glitters were examined: 'Jewel - Cherry Red', 'Stardust - Lime Green', 'Jewel-Oasis Blue', 'Jewel – Brilliant Bronze', 'Hologram White', 'Bubblegum' and 'Stardust Yellow'. The first combination made was: 'Hologram White', 'Lime green', 'none', and the combination of 'Lime Green' with 'Hologram White'. The second combination was: 'Oasis Blue', 'Lime Green', 'Bubblegum', and the combination of 'Bubblegum' with 'Hologram White'. The third combination was: 'Lime Green', 'Stardust Yellow', 'Bubblegum', and 'Oasis Blue'. The fourth combination was: 'Lime Green', 'Brilliant Bronze', the combination of 'Oasis Blue' with 'Brilliant Bronze', and the combination of 'Cherry Red' with 'Brilliant Bronze'. The fifth combination was: 'Lime Green', 'Oasis Blue', 'Cherry Red', and 'Bubblegum'. All glitters used in this study were of the company 'Rainbow Dust'.
The different colors and number of glitters found in the indoor enclosure, were documented, and compared to the actual color of the glitter examined in the laboratory of the Zoo. Resulting in the usage of the color-combination of the non-consumable-nontoxic glitters: 'Cherry red', 'Lime Green', 'Oasis Blue', and 'Bubble-Gum'.

Fecal examination and digestion rate

Macroscopic examination

From the 1st of April until the 27th of June 2018 a total of 281 fecal samples were obtained from our study group and control groups. All fecal samples derived from the study population were examined on the type and number of glitters in the sample, the digestion rate (1-7), the freshness of the sample (1-5), the color of the feces, the Waltham-score, the possibility of contamination of the sample, and the pH. The information above was mostly collected by one observer and examined in the laboratory of the Zoo. It was made sure that if another observer collected and examined the fecal samples, this individual received the 'fecal sampling form' and the 'fecal examination protocol' and was trained to collect and investigate the samples to minimize the possibility of observer bias. In May and June of 2018, the fecal samples were also investigated in the 'Universitair Veterinair Diagnostisch Laboratorium' (UVDL) for digestive enzymes, free fatty acids, fat, muscle, and starch. These examinations were obtained by one single observer, which was trained to do so following the protocols used at the UVDL. In the followed protocol, the safety measures, the amount of feces, the Lugol-solution, the Sudan III-solution, and the amount of Zymoral® used as a control is described.

Microscopic examination

The Microscopic examination of the fecal samples were performed by one single observer, who was trained by and followed the protocols of the UVDL. Before microscopic examination of the fecal sample, the pH would be determined. One gram of fecal sample would be placed into a mortar and grinded with a pestle to a pasty fecal consistency. Distilled water would slowly be added until the consistency of buttermilk was reached. This step was skipped whenever the fecal sample already had a watery consistency. The pH was measured using universal pH paper or the 'Duotest pH 1-12' and in the pH range was between the 5.0 and 8.0, the 'Duotest pH 5.0-8.0' was used.
After the determination of the pH, the microscopic slide was prepared with the same diluted fecal sample. Two droplets of diluted feces were placed on the microscopic slide using the pestle. One droplet of feces was surrounded by two droplets of Sudan III. A covering glass was placed carefully over the fecal droplet with the two Sudan III droplets. The Sudan III was prepared in the laboratory by combining 10mg Sudan III with 10mL of 96%-alcohol and then filling the bottle up to a 100mL with glacial acetic acid. The other droplet of feces was mixed with one droplet of Lugol-solution. The Lugol-solution consisted of 0.5grams of Iodine, 1 gram of Potassium-Iodide dissolved in 150mL with distilled water. A cover glass was also placed on top of the fecal droplet and Lugol-solution droplet. After which the Sudan III side of the microscopic slide was heated above a low flame. The heating process was stopped whenever tiny bubbles occurred amongst the sides of the cover glass of the

Sudan III cover glass. After which, the microscopic slide rested for 1.5 minutes before examination under a microscope. Both over glasses are examined with a microscope (x400). The cover glass covering the Lugol-combination is examined throughout the entire field for the number of muscle fibers and starch in the field. The cover glass covering the Sudan-combination is examined throughout 20 crosslinking fields, starting from the middle of the cover glass. Fat will be visible as little red droplets, Starch will be visible as purple dark blue or black material, muscle fibers will be visible by specific transverse fibers, and fatty acids will be visible as different shapes and sized needle shaped colorless crystals.

Digestive enzymes

To investigate the digestive enzymes a negative control sample will be made with Zymoral® following a protocol from the UVDL, used to determine the number of digestive enzymes in dogs and cats. One gram of Zymoral® contains 20.000 USP units' lipase, 100.000 USP units' protease, and 100.000 USP units' amylase. The amount of 0,02grams of Zymoral® is solved in 20mL of distilled water. A protein-plate is synthetized by covering a protein agar plate with acetic acid (5%) and a starch-plate is synthetized by covering a starch agar plate with the earlier mentioned Lugol-solution. In each of the two plates six wells will be formed to pipette exactly 20 microliters of sample- or control fluid in. One gram of cooled feces (max 4°C for max. 5 days) is combined with 9mL of Phosphate-buffered Saline (PBS). The sample combination was rotated slowly for one hour. After which it was centrifuged at 1000 for 10 minutes. Then 20microliters of supernatant was pipetted into one of the six formed wells on both plates. One control sample of Zymoral®-solution will be pipetted into one of the six wells of each plate. After this both plates will be put in the laboratory stove at 37 °C for nineteen hours. After which, the results can be calculated after measuring the diameter of the illumination around the perforated wells. Meaning the diameter of the illumination minus the original diameter of the wells (5millimeters) will result in the enzyme activity expressed in millimeters (mm). The results will be given as the diameter of the illumination(mm), reflecting the enzyme activity of the enzymes amylase on the starch-plate and protease on the protein-plate.

Infectious agents

To determine if infectious agents were of any influence on the intermitting diarrheal symptoms, multiple samples were investigated by a specialist in the laboratory of the Zoo on 3 following days (10^{th} of April until the 12^{th} of April 2018). A total of 14 fecal samples were collected and examined. The fecal samples were fixated in sodium acetate-acetic acid-formalin (SAF) and carefully mixed by inversion. The bacteriologic examination protocol of the laboratory of the Zoo was followed by the well-trained and educated Zoological laboratory analyst. Each fecal sample was inoculated onto different media agar plates, placed in stoves of 37°C and 25°C, and examined for bacterial growth after specific time periods.

Microbiome

Fecal samples of the study group were collected over the periods 19^{th} – 21^{st} of May and 10^{th} -11^{th} of June 2018. A total of 12 samples were collected of 4 individuals. After collection, the samples were conserved at -80 °C, and transported frozen to the 'BaseClear' facility in Leiden, the Netherlands. 'BaseClear' specializes in the development of microbial genomics by working intensively with pharmaceutical and biotechnology companies. At 'BaseClear' an experienced and trained laboratory technician used the '*Quick*-DNA Kit' of the 'Zymo Research' kits to purify and isolate the DNA from the biological samples using a specific protocol. A profiling service based on Next-Generation Sequencing (NGS) technology is used to determine the composition of bacteria, Archaea, and fungi. For the profiling of the bacteria, 16S rRNA gene (V3-V4) profiling primers were used. For the profiling of the fungi, 28S rRNA primers using 'Internal transcribed spacer' (ITS). Bacteria amplification is

accomplished by 16S rRNA gene (V3-V4) PCR amplification. Simultaneously, the barcoding and library preparation (1st step PCR and 2nd PCR) for Illumina sequencing is run, including quality control and quantification. Illumina MiSeq PE300 can deliver 10.000 reads for microbial profiling. A read is another way of stating that a rRNA strand is recognized. Overlapping pseudo-reads will be recognized and merged with the initial read. Subsequently, each read will be aligned against a 16S rRNA gene database for taxonomic classification. BaseClear uses the unique online genome browser tool 'Genome Explorer' to interpret the findings.

Statistical Methods

The aim of this study is to determine the possible correlation between the symptoms of intermitting diarrhea to the diversity in microbiome, the plausibility of digestion problems (with or without a determination of a causal disease), the presence and/or ratio of certain micro-organisms, and dietary factors. The microbiome data was fabricated with the module 'Genome Browser' by the medical laboratory 'BaseClear'. The statistical analysis was made with SPSS statistical software. Comparison between groups was made using the ANOVA-test, while the comparison between two specific conditions were compared with the independent T-test, to determine whether there was a significant difference between the two factors. A *P*-value of <0.05 was taken to indicate statistical significance. The microbiome has been reformed into percentages or 10log (+1) to normalize the values. Comparisons of the digestive enzymes have been calculated with the Wilcoxon paired T-test and the Mann-Whitney U-test. Figures were made with SPSS or Excel.

Results

Study Group

The study population consists of 4 individual females with the ages 19, 14, 2, and 1.5 years old, referred to according to age as individual 1,2,3, and 4. A total of 249 fecal samples were collected from the study group. A total of 31 fecal samples were collected from the control groups. A *P*-value of <0.05 was taken to indicate statistical significance. The mean Waltham score of the eldest adult (individual No. 1) *M.nigra* of the study group was 4.47 (SD = 0.80). The mean Waltham score of the youngest adult (individual No. 2) *M.nigra* was 3.47 (SD = 0.84). The mean Waltham score of the eldest juvenile (individual No. 3) *M.nigra* was 3.64 (SD = 0.88). The mean Waltham score of the youngest juvenile (individual No.4) *M.nigra* was 3.63 (SD = 0.73). The Waltham scores examined with the ANONA-test, showed a significant difference (*P*<.0001) between individuals of the study group. The Waltham-score of the eldest adult *M.nigra* showed a significant difference with the 3 other individuals. The others showed no significant difference to one another. These comparisons were done with the independent-samples t-test.
The mean pH of individual No. 1 was 7.22 (SD = 0.57). The mean pH of individual No. 2 was 6.76 (SD = 0.39). The mean pH of individual No. 3 was 6.73 (SD = 0.36). The mean pH of individual No. 4 was 6.95 (SD = 0.36) (Table 1). The pH-scores within the *M.nigra* group compared with the ANOVA-test with each other showed a significant difference (*P*<.0001). The pH of the eldest adult *M.nigra* showed a significant difference with the 3 other individuals. The pH of the eldest juvenile also showed a significant difference with the youngest juvenile(*P*=.008). These comparisons were done with the independent-samples t-test (Table 1).

Table 1. Measured Waltham-scores, pH and the comparison of the digestion rates

	1 Mean(SD)	2 Mean(SD)	3 Mean(SD)	4 Mean(SD)	Group Mean(SD)
Waltham:	4.47(0.80), *N* = 37	3.47(0.84), *N* = 36	3.64(0.88), *N* = 60	3.63(0.73), *N* = 64	3.76(0.88), *N* = 197
pH:	7.22(0.57), *N* = 22	6.76(0.39), *N* = 24	6.73(0.36), *N* = 38	6.94(0.36), *N* = 45	6.9(0.44), *N* = 129
Digestion rate 1:	5.18(0.82), *N* = 17	2.4(0.83), *N* = 15	2.24(0.50), *N* =34	2.40(0.46), *N* = 30	2.83(0.36), *N* = 96
Digestion rate 2:	5.00(0.84), *N* = 18	2.37(0.75), *N* = 16	2.83(0.59), *N* = 35	2.58(0.65), *N* = 31	3.07(0.38), *N* = 100

Data are mean (SD) or number. N stands for number of fecal samples. Individual measurements of the study group: 1= the oldest adult female; 2 = The youngest adult female; 3 = the oldest juvenile female; 4 = the youngest juvenile female; Digestion rate 1 = The digestion rate of the fecal samples scored in the indoor enclosure; Digestion rate 2 = The digestion rate of the fecal samples scored in the laboratory.

The digestion rate scored in the indoor enclosure is compared to the digestion rate scored in the laboratory to determine if it is advisable to determine this macroscopically measured factor should be investigated in future research. The mean digestion rate of the fecal samples derived from the eldest adult of the *M.nigra* group, scored in the indoor enclosure was 5.18 (SD = 0.82). The mean digestion rate of the fecal samples derived from the youngest adult of the *M.nigra* group, scored in the indoor enclosure was 2.4 (SD = 0.83). The mean digestion rate of the fecal samples derived from the eldest juvenile of the *M.nigra* group, scored in the indoor enclosure was 2.24 (SD = 0.5).The mean digestion rate of the fecal samples derived from the youngest juvenile of the *M.nigra* group, scored in the indoor enclosure was 2.40 (SD = 0.46). The mean digestion rates in the indoor enclosure compared to one another with the ANOVA-test did differ significantly (*P*<.0001). The mean digestion rate in the indoor enclosure derived from the eldest adult showed a significant difference with the 3

other individuals. The others showed no significant difference to one another. These comparisons were done with the independent-samples t-test.
The mean digestion rate of the eldest adult of the *M.nigra* group, measured macroscopically in the laboratory, was 5.00 (SD = 0.84). The mean digestion rate of the youngest adult of the *M.nigra* group, measured macroscopically in the laboratory, was 2.37 (SD = 0.75). The mean digestion rate of the eldest juvenile of the *M.nigra* group, measured macroscopically in the laboratory, was 2.83 (SD = 0.59). The mean digestion rate of the youngest juvenile of the *M.nigra* group, measured macroscopically in the laboratory, was 2.58 (SD = 0.65). The mean digestion rate measured in the laboratory in the *M.nigra* group compared with the ANOVA test with each other did differ significantly (*P*<.0001). The mean digestion rate in the laboratory of the eldest adult of the *M.nigra* group showed a significant difference with the 3 other individuals. The others showed no significant difference to one another. These comparisons were done with the independent-samples t-test (Table 1).

Control groups

The control groups consist of fecal samples from different *M.mulatta* enclosures at the BPRC. Individual samples were collected from both healthy groups (*N*=20) and groups with diarrheal (*N*=1) symptoms. Secondly some group samples (min >3 fecal samples) were collected into one tube, taken from different enclosures both healthy(*N*=3) and with diarrheal(*N*=8) symptoms. The mean Waltham score of the samples from the healthy control individuals was 2.5. The Waltham score of the sample from our diarrhea individual was 5. The mean Waltham score of our healthy samples from the control group was 3.5. The Waltham score of our diarrhea samples from the control group was 3.8. The mean pH of the sample from our healthy control individual was 6.9. The pH of the sample from our diarrhea individual was 6.5. The mean pH of the samples from the healthy control group was 7.6. The pH of our diarrhea samples from the control group was 6.4 (Table 2).

Table 2. Comparison of continuous variables between different control groups

	Control sample healthy individual Mean(SD)	Control sample diarrhea individual (*N=1)* Mean	Healthy group control sample (*N=3)* Mean(SD)	Diarrhea group control sample (*N=8)* Mean(SD)
Waltham:	2.5(1.13), *N* = 13	5.0	3.5(1.32)	3.8(0.53)
pH:	6.9(0.56), *N* =12	6.5	7.6(0.69)	6.4(0.48)
Digestion rate 1:	1(0.00), *N* = 20	3	3(3.46)	3(2.25)
Digestion rate 2:	2(1.04), *N* = 11	3	4(3.06)	4(1.85)

Data are mean (SD) or number. N stands for number of fecal samples. Control samples collected from groups of *M.mulatta* at the BPRC. 'Control sample healthy individuals' = individual fecal samples collected from healthy groups at BPRC. 'Control sample diarrhea individuals' = individual fecal samples collected from groups with diarrheal symptoms at BPRC. 'Healthy group control sample'= group fecal samples collected from healthy groups at BPRC. 'Diarrhea group control sample'= group fecal samples collected from groups at BPRC with diarrheal symptoms.; Digestion rate 1 = The digestion rate of the fecal samples scored in the indoor enclosure; Digestion rate 2 = The digestion rate of the fecal samples scored in the laboratory.

The mean digestion rate in the enclosure of the sample from our healthy control individual was 1. The digestion rate in the enclosure rate of the samples from the indoor enclosure of the sample from the individual with diarrheal symptoms was 3. The mean digestion rate in the enclosure of the healthy group samples was 3. The digestion in the enclosure of the diarrheal group samples was 3. The digestion rate measured in the laboratory of the sample from our healthy control individuals was 2. The digestion rate measured in the laboratory of the sample from our diarrhea individual was 3. The mean digestion rate measured in the laboratory of our healthy group samples was 4. The digestion measured in the laboratory of the diarrheal group samples was 4 (Table 2).

In the following section the healthy samples of the *M.mulatta* population (including both individual- and group samples) are compared with the diarrheal samples of the *M.mulatta* population (including both individual-and group samples). The mean Waltham score of the healthy control group samples 2.75 (SD = 0.87). The mean Waltham score of the diarrheal control group samples was 3.94 (SD = 0.63). The mean pH of the healthy control group samples 7.00 (SD = 0.64). The mean pH of the diarrhea control group samples was 6.40 (SD = 0.45) (Table 3).

Table 3. Comparison of continuous variables between control healthy samples and control diarrheal samples from the *M.mulatta* control groups.

	Control healthy samples Mean(SD)	Control diarrheal samples Mean(SD), (*N = 9)*
Waltham:	2.75(0.87), *N* = 14	3.94(0.63)
pH:	7.00(0.64), *N* =16	6.40(0.45)
Digestion rate 1:	2.43(2.59), *N* = 29	3.22(2.11)
Digestion rate 2:	2.00(1.71), *N* = 14	3.89(1.76)

Data are mean (SD) or number. N stands for number of fecal samples. Control samples collected from groups of *M.mulatta* at the BPRC. 'Control healthy samples'= individual and group fecal samples collected from healthy groups at BPRC. 'Control diarrhea samples' = individual and group fecal samples collected from groups with diarrheal symptoms at BPRC.; Digestion rate 1 = The digestion rate of the fecal samples scored in the indoor enclosure; Digestion rate 2 = The digestion rate of the fecal samples scored in the laboratory. *P*-value for Independent T-test comparing distribution between populations.

The mean digestion rate of the samples from the indoor enclosure of the healthy control group samples was 2.43 (SD = 2.59). The mean digestion rate of the samples from the indoor enclosure of the diarrhea control group samples was 3.22 (SD = 2.11). The mean digestion rate measured in the laboratory of the healthy control group samples was 2.00 (SD = 1.71). The mean rate digestion measured in the laboratory of the diarrhea control group samples was 3.89 (SD = 1.76) (Table 3).
To compare the Waltham-score and the pH of the *M.nigra* population with the *M.mulatta* population, a total of 269 samples is used. Both groups contain both 'healthy' as 'diarrheal' fecal samples (Table 4).

Table 4. Comparison of continuous variables between *M.nigra* group and control *M.mulatta* group.

Variable	*M.nigra*, Mean(SD)	Control group *M.mulatta*, Mean(SD)	*P*-Value*
pH	6.88(0.46), *N* = 243	6.79(0.64) *N* = 25	.346
Waltham	3.89(2.18), *N* = 244	3.22(0.98) *N* = 23	.005
pH without urine contamination	6.87(0.44), *N* = 210	6.79(0.64) *N* = 25	.389

P-value for Independent T-test comparing distribution between the *M.nigra* population and the *M.mulatta* population.

The Waltham score of the *M.nigra* population compared with control *M.mulatta* population showed a significantly higher Waltham-score (*P*=.005). The samples without the possible contamination of urine of the *M.nigra* population, are compared with those the *M.mulatta* population and did not show any significant difference (*P*=.389).

The mean pH of the *M.nigra* group was 6.88 (SD = 0.64). The mean pH of the healthy control group *M.mulatta* was 7.00 (SD = 0.64). The mean pH of the diarrhea control group *M.mulatta* was 6.40 (SD = 0.46). The mean Waltham score of the *M.nigra* group was 3.89 (SD = 2.18). The mean Waltham score of the healthy control group *M.mulatta* was 2.75 (SD = 0.87). The mean Waltham score of the diarrhea control group *M.mulatta* was 3.95 (SD = 0.63). The pH of the *M.nigra* group compared with healthy control *M.mulatta* group did not differ significantly (*P*=.346). The pH of the *M.nigra* group compared with diarrhea control *M.mulatta* group did differ significantly (*P*=.002). The Waltham score of the *M.nigra* group compared with the healthy control *M.mulatta* group did not differ significantly (*P*=.052). The Waltham score of the *M.nigra* group compared with the diarrhea control *M.mulatta* group did not differ significantly (*P*=.994) (Table 5).

Table 5. Comparison of continuous variables between *M.nigra* group and control *M.mulatta* group.

Variable	*M.nigra*, Mean(SD)	Control group *M.mulatta* healthy, Mean(SD)	*P*-Value*
pH	6.88(0.64), *N* = 243	7.00(0.64), *N* = 16	.314
Waltham	3.89(2.18), *N* = 244	2.75(0.87), *N* = 14	.052
Variable	*M.nigra*, Mean(SD)	Control group *M.mulatta* diarrhea, Mean(SD),	*P*-Value*
pH	6.88(0.64), *N* = 243	6.40(0.46), *N* = 9	.002
Waltham	3.89(2.18), *N* = 244	3.95(0.63), *N* = 9	.994

P-value for Independent T-test comparing distribution between populations

Fecal identification methods

The fecal identification methods were examined in two steps.
First the non-consumable, nontoxic glitters, the consumable glitters, and the beads examined in the laboratory on color retention and the reflection of colors in fecal samples. The second investigation was to determine the gastrointestinal passage time, and the number of glitters and color-combination needed to maximize the efficacy of this marker method.
In the first investigation 3 groups were determined: Plastic beads, consumable glitters, and non-consumable-nontoxic glitters. The glitter colors were examined after 1 day (Test 1), 2 days (Test 2) and after 22 days (Test3). Table 6., Shows the colors that were seen by examining the glitters, within the fecal samples.

Table 6. The color variety of the different kind of glitters

Color administered	Color outcome Test 1	Color outcome Test 2	Color outcome Test 3
Purple	black	black, purple	black, purple
Hologram white	all colors	all colors	all colors
Red	red	red	red
Stardust Lime	lime green	lime green	lime green
Gold	gold	gold	gold
Silver	silver	silver	zilver
Apple Green	green	green	green
Ocean Blue	blue	blue	blue
Stardust Yellow	yellow, orange, green	yellow, orange, green	yellow, orange, green
Stardust Bubblegum	pink, green, yellow, orange	pink, green, yellow, orange	pink, green, yellow, orange

Colors that were seen while examining the glitters in the fecal samples

Secondly, the color retention was investigated and compared between the plastic beads (*N*=8), the consumable glitters (*N*=6), and the non-consumable-nontoxic glitters (*N*=5). The glitters and beads were all placed in fecal samples with differences in Waltham-scores, pH, and freshness. No significant differences were found between the 3 categories of fecal samples when compared to the color retention of the beads nor glitter-groups.
On T1 there is a difference noticeable between the number of glitters found in the fecal samples. Interestingly, the colors of the consumable glitters have all been reduced to shimmering fecal samples. On T3 only 2 consumable glitter colors showed a positive result: ‘Ocean Blue’ showed a shimmering and ‘Gold’ showed a green/orange colored glitter, which could possibly be a false positive. All non-consumable-nontoxic glitters remained visible with no visible degradation in number of glitters (Table 7).

Table 7. Measured color variation, recognition, and number of glitters

Glitters, color	Color recognition (T0)	Color recognition (T1) (number of glitters)	Color recognition (T2) (number of glitters)	Color recognition (T3) (number of glitters)
Purple	no	no(0)	no(0)	no(0)
Hologram white	yes, rainbow color	partial, blue, green, orange, purple(4)	partial, green, yellow, white(4)	partial, white(4)
Red	partial, red	no(0)	yes, red(1)	no(0)
Stardust Lime	yes, green	yes, green(4)	yes, green, yellow(4)	yes, green, yellow(4)
Gold	yes, gold	no(0+1)	no(0+1)	yes, green + orange(1+0)
Silver	partial, silver	no(0+1)	no(0+1)	no(0)
Apple Green	partial, dark green	no(0+1)	yes, green, (1+1)	no(0)
Ocean Blue	yes, dark blue	no(0+1)	no(0+1)	no(0+1)
Stardust Yellow	yes, rainbow yellow	yes, yellow(4)	yes, yellow(4)	yes, green, yellow(4)
Stardust Bubblegum	yes, bubblegum pink	yes, bubblegum pink(4)	yes, bubblegum pink(4)	yes, bubblegum pink(4)
Jewel Ocean Spray	yes, rainbow	yes, blue(4)	yes, blue(4)	yes, blue(4)

Descriptive summary of the laboratory results of the comparison of the color recognition and number of glitters seen over time, in fecal samples containing consumable or non-consumable-nontoxic glitters. Color recognition: T0 = 8-4-2018; T1=9-4-2018; T2=10-4-2018; T3=30-4-2018. Number of glitters: 0=none, 1=a few, 2=several, 3=many, 4=abundant, 0+1 = no glitters visible, but a shimmering, 1+0 = a few glitters, no shimmering (only if a shimmer was to be expected).

Of the 8 different type of colored plastic beads, 4 showed color retention and became less recognizable in the fecal samples. The colors white, shiny white, silver, and black remained the exact color during this test (Table 8). However, the differences between white, and shiny white were not easily seen.

Table 8. Measured color recognition (and retention of color) in plastic beads

Bead color	Color recognition(T0)	Color recognition(T1) (Color retention)	Color recognition(T2) (Color retention)	Color recognition(T3) (Color retention)
white	white	white(no)	white, creme(no)	white(no)
pink	pink	light pink(yes)	light pink(yes)	light pink(yes)
pearl	pearl	shiny white(no)	shiny white(no)	shiny white(no)
silver	silver	silver(no)	silver, yellow(no)	silver(no)
green	green	light green(yes)	light green(yes)	light green(yes)
purple	purple	light pink(yes)	light pink(yes)	light pink(yes)
blue	blue	light green(yes)	light green(yes)	white, green, blue(yes)
black	black	black(no)	black(no)	black(no)

Descriptive summary of the laboratory results of the comparison of the color recognition (and the retention thereof) seen over time, in fecal samples containing consumable or non-consumable-nontoxic glitters. Color recognition: T0 = 8-4-2018; T1=9-4-2018; T2=10-4-2018; T3=30-4-2018. Color retention: 'yes' = color is fading or less recognizable within the fecal sample; 'No' = color is not fading and still recognizable within the fecal sample.

In the second part of the search for the most accurate fecal identification method for our study group, the gastrointestinal passage time, number of glitters, and color-combinations needed to be examined. The gastrointestinal passage time was discovered to be within the range of <24hours to 3 days. The glitters were clearly visible in all 4 individuals after consuming ½ teaspoon. The number of glitters needed for accurate identification was discovered by slowly heightening the dosages starting with an est. of 1/5th of a teaspoon. The gastrointestinal passage time was measured by administering glitters to all individuals on one day and measuring the fecal samples the following 4-5 days. This has been repeated two times, giving the same results. To investigate the best color-combination,

multiple combinations were made, giving the following results:
Combination 1 ('Hologram White', 'Lime green', 'none', and the combination of 'Lime Green' with 'Hologram White'): resulted in the conclusion that all individuals needed to be fed a different color of glitters. This because every individual has a different gastrointestinal passage time, and they steal each other's rice balls making it harder to identify the feces of one individual when two have ingested the color 'Lime green'.
Combination 2 ('Oasis Blue', 'Lime Green', 'Bubblegum', and the combination of 'Bubblegum' with 'Hologram White'): The colors 'Oasis Blue' and 'Lime Green' were clearly visible and distinguishable from the other used colors. The color 'Hologram White' was distinguishable because it was the only glitter that reflected purple in a fecal sample.
Combination 3 ('Lime Green', 'Stardust Yellow', 'Bubblegum', and 'Oasis Blue'): The color 'Stardust Yellow' was not easily distinguished from the colors 'Bubblegum' and 'Lime Green', since it reflected the colors yellow, green, and orange. The colors 'Bubblegum' and 'Lime green' were distinguishable from one another.
Combination 4 ('Lime Green', 'Brilliant Bronze', the combination of 'Oasis Blue' with 'Brilliant Bronze', and the combination of 'Cherry Red' with 'Brilliant Bronze'): Even though it was visible that there was a glitter within the fecal samples of the color 'Brilliant Bronze', the color itself was not clearly visible. The combination samples were a good identification marker in the laboratory examination, but not so much in the indoor enclosure.
Combination 5 ('Lime Green', 'Oasis Blue', 'Cherry Red', and 'Bubblegum'): All uses colors were distinguishable from one another in the indoor enclosure and in the laboratory. The glitter 'Lime Green' reflected the colors green and yellow in the fecal samples. The glitter 'Oasis Blue' reflected the colors black and blue in the fecal samples. The glitter 'Cherry Red' reflected red in the fecal samples and the color 'Bubblegum' reflected the colors pink, orange, and yellow in the fecal sample. In the end the colors 'Lime green', 'Oasis Blue', 'Cherry Red', and 'Bubblegum', were found the most effecting markers to identify the fecal samples of the study group.

Digestion rate

An indication of the digestion rate is measured by examining the fecal samples macroscopically, microscopically, and by investigating the enzyme activity in diameter (mm). The comparison between the Waltham-score and the pH is made to determine if the pH can be a prognostic factor for a higher Waltham-score. The pH was significantly higher in the samples with a Waltham-score of 4.0 or higher (P=.026) (Table 9).

Table 9. Comparison of continuous variable between Waltham-score ≤ 3.5 and Waltham-score ≥ 4.0

Variable	Waltham-score ≤ 3.5 Mean(SD)	Waltham-score ≥ 4.0, Mean(SD)	*P*-Value*
pH	6.81(0.41), *N* = 143	6.95(0.56) *N* = 119	.026

P-value for Independent T-test comparing distribution between the group: 'Waltham-score ≤ 3.5 = all 'healthy' samples and the group: Waltham-score ≥ 4.0 = all the "diarrheal samples". All samples included (control groups and study group)

Microscopic examination:

The microscopic examination was performed on samples from the study group (*N*=13) and the control group (*N*=21). The Mean(SD) measurements can be found in Table 10. The amount of muscle fibers found in these fecal samples was 0, and therefore left out.

Table 10. Measured Mean(SD) of Starch, Fat, and Fatty acids within the study group.

Animal	Fatty acids, Mean(SD)	Fat, Mean(SD)	Starch, Mean(SD)
1	0.3(0.5), N = 4	8.8(6.2), N = 4	147.5(179.7), N = 4
2	0(0), N = 2	38.5(0), N = 2	186.5(252.4), N = 2
3	4.0(2.8), N = 2	30.5(13.4), N = 2	160.5(201.5) N = 2
4	2.0(1.8), N = 5	68.2(104.3), N = 5	708.4(734.2), N = 5
Total	1,4615 N = 13	39,5385 N = 13	371,2308 N = 13

The Mean(SD) is derived from the microscopically measured numbers of Fatty acids, Fat, and Starch present in the fecal samples of the M.nigra population. 'Animal No. 1' = the eldest adult female; 'Animal No. 2' = the youngest adult female; 'Animal No. 3' = the eldest juvenile female; 'Animal No. 4' = the youngest juvenile female.

The number of fat in the fecal samples from the *M.nigra* population was substantially lower than the number of fat in the fecal samples from the *M.mulatta* population (Table 11). Muscle fibers were examined, but none were found in the samples and therefore not mentioned.

Table 11. Comparison of continuous variables between *M.nigra* group and control *M.mulatta* group.

Variable:	*M.nigra*, Mean(SD), *(N=13)*	Control group *M.mulatta*, Mean(SD), *(N=21)*	*P*-value
Fatty acids	1.46(1.81)	1.67(4.29)	.872
Fat	39.5(68.64)	79.45(40.52)	.028
Starch	251.42(308.03)	955.57(603.76)	.225

P-value for Independent T-test comparing distribution between both populations (*M.nigra vs. M.mulatta). Both populations include diarrheal samples and 'healthy' samples.*

Digestive enzymes

The Mean (SD) of the amylase and protease enzyme activities of the *M.nigra* group can be compared between the months May and June, by selecting the best four samples from May. This way both months will have one fecal sample representing each individual. The sample numbers: 9, 10, 11,12 were chosen to represent the month May and the sample numbers: 278, 279. 280, and 281 were chosen to represent the month June. All used samples were first corrected for the measurement deviation of the plates (Table 12). No significant differences of enzyme activity were found, when comparing the fecal samples of the study group in May and June.

Table 12. Comparison of corrected plate values of digestive enzymes of the study group between May and June 2018.

Corrected with the neg. controls of the test	May, Mean(SD), (*N* = 4)	June, Mean(SD), *(N*= 4)	P-Value
Amylase	3.5(1.7)	3.0(1.4)	.854
Protease	5.5(1.3)	6.0(0.0)	.414

P-value for Wilcoxon paired T-test: Study group fecal samples from 'May' represented by samples: 9, 10, 11, 12; and 'June' represented by samples: 278, 279, 280, 281.

The Mean (SD) of the amylase and protease enzyme activities in May, 2018 of the *M.nigra* group were compared with the Mean(SD) of the enzyme activities of the same enzymes of the *M.mulatta* group. The protease activity in the fecal samples of the *M.mulatta* group were significantly lower compared to the *M.nigra* group in May 2018 (Table 13).

Table 13. Comparison of continuous variables between the *M.nigra* group and the control *M.mulatta* group.

Variable	*M.nigra* May, Mean (SD) *N* = 14	Control group *M.mulatta*, Mean(SD) *N* = 24	*P*-Value*
Amylase	4.1(3.3)	3.7(4.2)	.584
Protease	8.3(4.7)	4.1(4.2)	.007

P-value for Mann-Whitney U comparing distributions between the study population and the control population

The Mean (SD) of the amylase and protease enzyme activities in June, 2018 of the *M.nigra* group were compared with the Mean(SD) of the enzyme activities of the same enzymes of the *M.mulatta* group. No significant differences were found (Table 14).

Table 14. Comparison of continuous variables between the *M.nigra* group and the control *M.mulatta* group.

Variable	*M.nigra* June, Mean (SD) *N* = 5	Control group *M.mulatta*, Mean(SD) *N* = 24	*P*-Value*
Amylase	5.2(1.3)	3.7(4.2)	.147
Protease	2.0(0.7)	4.1(4.7)	.400

P-value for Mann-Whitney U comparing distribution between populations

Microbiome

The results of the microbiome of the *M.nigra* of the study group are compared on a level of Kingdom and Phylum. A total amount of 12 samples were examined, with a minimum of 2 samples per individual and a maximum of 4 samples per individual. 8 samples were collected in May 2018 and 4 samples were collected in June 2018. The found Kingdoms derived from the four *M.nigra* individuals are: Archaea, Bacteria, Eukaryota, and Fungi. The percentages derived from the averages of the total found numbers of DNA classified in Kingdom show that Bacteria and Unclassified are primarily found in the fecal samples (Table 15).

Table 15. Average percentages of the fecal differentiation on the level of Kingdoms.

	% per sample (*N*=12)	% per sample May (*N*=8)	%per sample in June (*N*=4)
Archaea	0,1	0,0	0,1
Bacteria	68,0	67,6	68,8
Eukaryota	0,0	0,0	0,0
Fungi	0,0	0,0	0,1
Unclassified	31,8	32,3	30,9

Results are in percentages of the mean samples.

The Archaea found in the fecal samples of the *M.nigra* group are of the Phylum: Euryarchaeota. The Eukaryota found in the fecal samples of the *M.nigra* group are of the Phylae: Ascomycota, Chlorophyta, Streptophyta. The Fungi found in the fecal samples of the *M.nigra* group are of the Phylae: Basidiomycota and unidentified fungi-groups. The Bacteria found in the fecal samples of the *M.nigra* group are of the Phylae: Actinobacteria, Bacteriodetes, Candidatus Saccharibacteria, Chlamydiae, Chloroflexi, Chrysiogenetes, Cyanobacteria, Deferribacteres, Deinococcus-Thermus, Elusimicrobia, Fibrobacteres, Firmicutes, Fusobacteria, Gemmatimonadetes. Lentisphaerae, Planctomycetes, Proteobacteria, Spirochaetes, Synergistetes, Tenericutes, Unclessified bacteria, unidentified bacteria, and Verrucomicrobia. The largest percentage of phyla in the bacterial microbiome of the *M.nigra* group are the Firmicutes and the Bacteroidetes. The major genera within the Firmicutes phyla included: *Ruminococcaceae* (27.4%), *Lachnospiraceae*(24.3%), *Clostridiaceae*(12.2%), *Lactobacillaceae*(6.5%), *Eubacteriaceae*(6.5%). The *Enterococcus* represented only 0.1% of the phyla Firmicutes.

The bacterial phylae have also been compared to the Waltham-scores of the individuals and the differentiation between the samples collected in May and June have been examined. Waltham-scores beneath 3.5 were categorized as 'normal feces' and samples above 3.5 were categorized as diarrheal samples. The 10log of the average numbers of phylae was used to normalize the average bacterial numbers. No significant differences were found between the bacterial phylae of all 12 samples. The pie charts of the 10log distribution of the bacterial phylae from the *M.nigra* individuals can be found in the attachments of this study (Attachment 5 of this study).

To examine if the microbiomes of the individuals showed any real significant difference between the months May and June, the Wilcoxon paired t-test was performed. For this test, the samples 3, 5, 10, and 11 were chosen to represent the microbiome of the study group in May 2018 and for June 2018 the samples 278, 279, 280, and 281 were chosen. No significant change was found in the microbiome of the study group between the months May and June 2018 (Table 16).

Table 16. Comparison of Microbiome between May and June

	May, Mean (SD), (*N* =4)	June, Mean (SD), *N* = 4	*P*-Value
Euryarchaeota	0.84(0.85)	1.43(0.77)	.237
Actinobacteria	2.71(0.07)	2.58(0.09)	.068
Bacteroidetes	3.99(0.05)	3.94(0.05)	.068
Chlamydiae	0.77(0.45)	0.81(0.37)	.999
Cyanobacteria	1.76(0.46)	1.77(0.05)	.999
Elusimicrobia	0.65(0.23)	0.92(0.09)	.068
Fibrobacteres	1.11(0.13)	1.30(0.14)	.109
Firmicutes	4.40(0.05)	4.19(0.50)	.999
Lentisphaerae	0.65(0.42)	1.04(0.31)	.144
Planctomycetes	1.27(0.73)	1.64(0.09)	.465
Proteobacteria	2.84(0.20)	2.77(0.06)	.715
Spirochaetes	3.34(0.28)	3.22(0.18)	.465
Synergistetes	0.64(0.23)	0.69(0.07)	.655
Tenericutes	1.44(0.37)	1.53(0.29)	.465

P-value for Wilcoxon paired T-test comparing distribution between the group: 'May 2018' and 'June 2018' for individuals 1, 2, 3, and 4.

The group differences per phyla are represented in *figure 1*. Even though, no significant differences found within the phyla between the months May and June, the Euryarchaeota, Planctomycetes, and the Lentisphaerae show the largest difference within the group (*figure 2*).

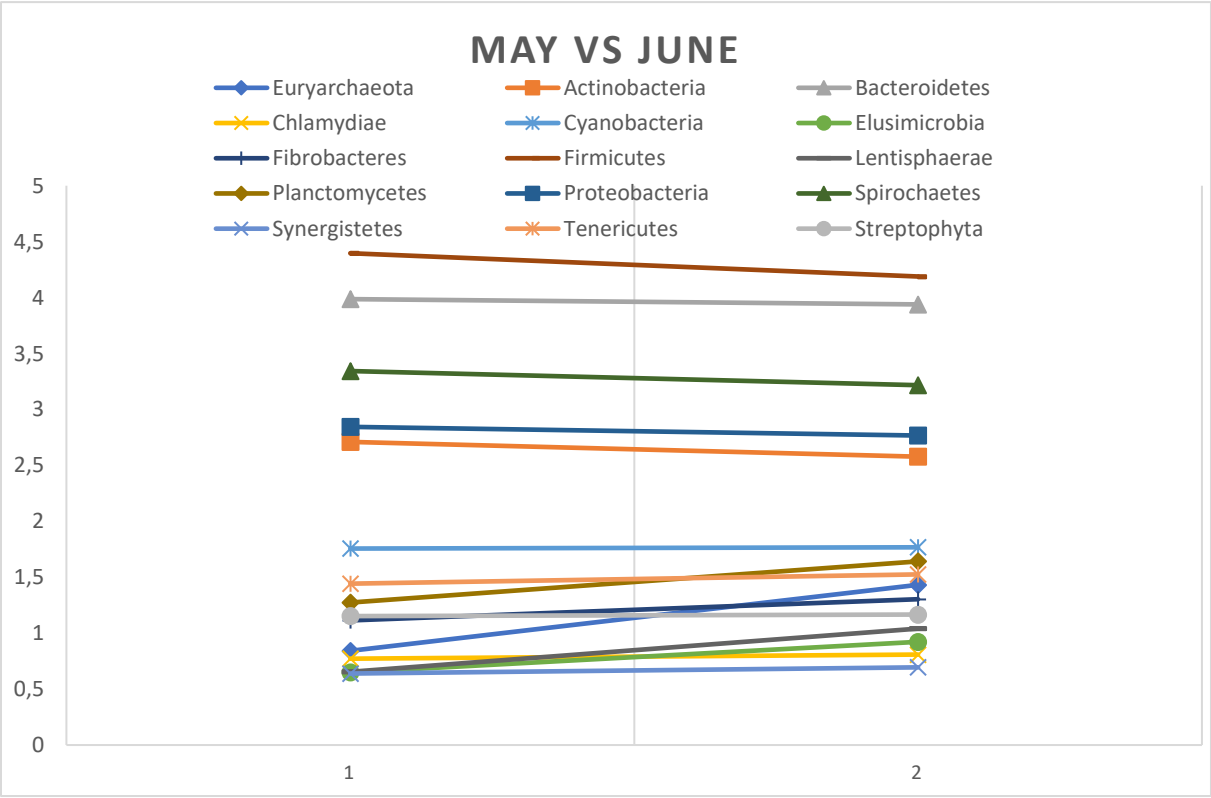


Figure 1: Paired t-test: Comparison of the bacteria phyla of the microbiome of the study population in May 2018 and June 2018.

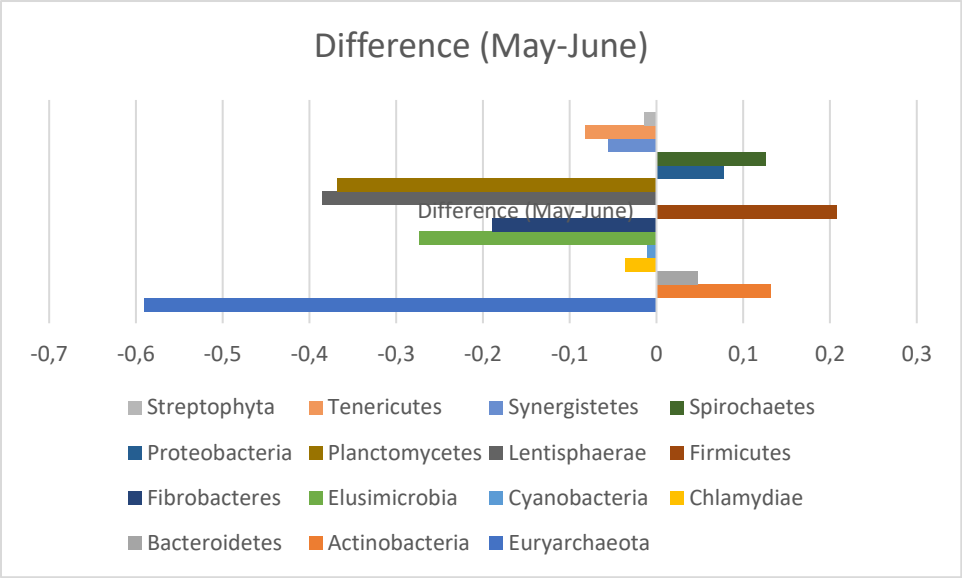


Figure 2: Paired t-test: Comparison of the bacteria phyla of the microbiome of the study population in May 2018 and June 2018.

To see if the group differences in phylae of the bacteria were derived from all the individuals, or whether it was mostly due to only one individual, the same figures were made per individual (Attachment 5 of this study). These figures show that individual 1 shows the largest difference in Cyanobacteria, followed by the Spirochaetes, and the Euryarchaeota. Individual 2 shows a large difference in Firmicutes, and smaller differences in Euryarchaeota and Tenericutes. Individual 3 shows a major difference in Euryarchaeota, Plantctomycetes, and Lentisphaerae. The largest differences in individual 4 are seen within the Cyanobacteria, Chlamydiae, and Streptophyta. Concludingly, the differences in the Euryarchaeota as seen in *figure 2*, are representative for 3 individuals, but the main differences are derived from the samples of individual 3. To compare the individual microbiome results all samples that were examined at 'BaseClear' were included and represented in percentage of phyla per sample. These percentages show large differences within the following phyla: the Actinobacteria, the Bacteriodetes, the Firmicutes, the Fibrobacteres, and the Spirochaetes.

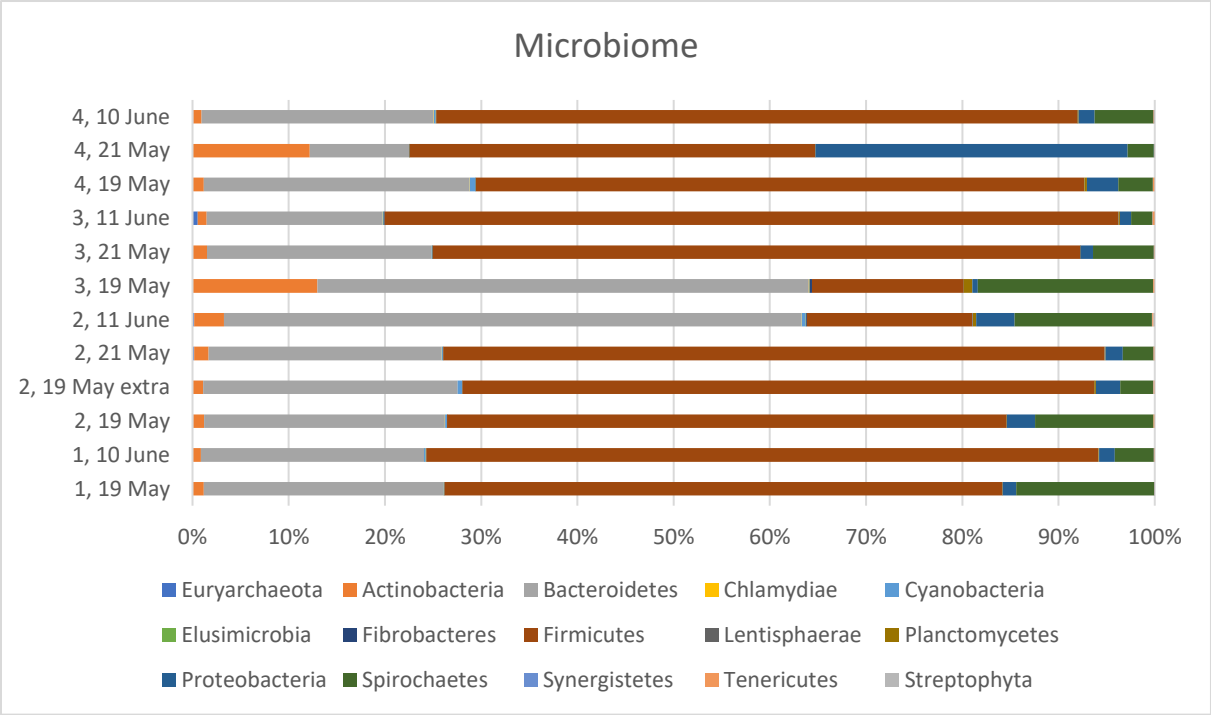


Figure 3: Overview of percentiles of bacteria phyla found in fecal samples of individual 1, 2, 3, and 4 from the study group on different dates in May and June 2018.

Infectious agents:

The laboratory of the Zoo examined 14 fecal samples of the study population. These samples had a freshness-score between 1 and 5 and an average Waltham-score of 4,9. Most samples were from individual 1 (*N=7);* one sample was from individual 2; and the others were marked as 'unknown'. Twelve samples resulted in Proteus nv. Bacteria. The remaining two samples had a multicultural overgrowth.

Discussion

Study population and control groups

Because the study population consists of four individuals the significance of many results must be interpreted with caution. The control groups unfortunately consisted out of a different species of *Macacas*. The genus *Macaca* consists of 22 species and 37 taxa. The evolutionary history of these groups is not completely clear, but the current proposal is that there are three monotypic species groups and four polytypic species groups comprising several species. The *M.mulatta* and *M.nigra* are according to the current beliefs not from the same polytypic species groups[25].

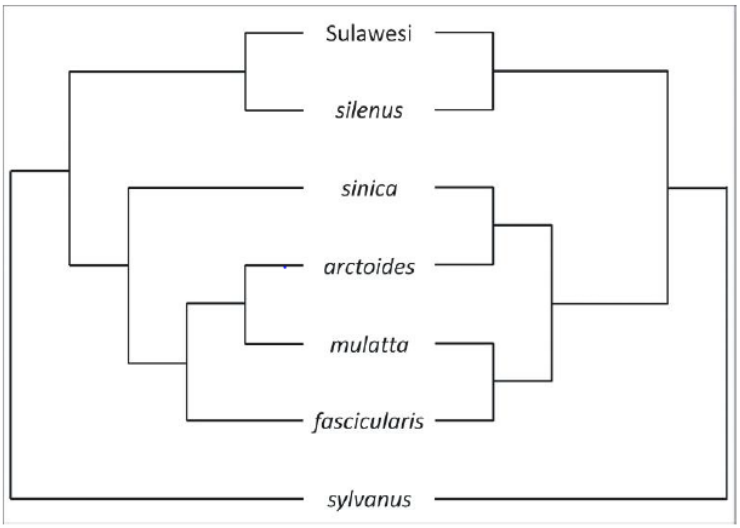


Figure 4 Evolutionary history of macaques and the Phylogenic relationships among macaque species groups based on mitochondrial (left) and Y chromosomal DNA (right) according to Bluemel et al. (2013)

However, the *M.mulatta* was genetically the closest species to our study population, which was available for participation at the time of this study. Information about the control groups enclosures, diets, and other external factors were documented because of the location they were kept. The BPRC facility also had groups of *M.mulatta* dealing with chronic intermitting diarrheal symptoms and groups without any clinical symptoms, which made those groups plausible control groups. Even though the environmental factors of the *M.mulatta* group were controlled within reason, they were not changed to meet the environmental factors of our study group. Furthermore, the *M.mulatta* is one of the most commonly used species in researches and therefore well known[26]. Therefore, this was the best possible group selection at the time of this study. In the future preferably more groups of *M.nigra* should be compared to have a larger population and the control groups should preferably be of the 'Sulawesi macaques group'[25].

Fecal identification methods

Markers are used as a fecal identification method for group-housed animals for years. Another usage for fecal markers in the past has been to determine the gastrointestinal transit time of individuals. Zoological facilities mention the usage of bakers' paste, Wilton paste food dye, Lake pigment dyes, Plastic Beads, different types of food coloring liquid (mostly green and blue), gel, blueberries, Millet, Cr-mordanted fiber, Co-EDTA, sesame seeds, nontoxic glitters in primates. Other possible markers for fecal identification are: sunflower seeds, digestible glitters, raw corn, poorly digestible grains, chromic oxide, biodegradable barrier tape, and vegetables[24,27,28]. It is preferable to use markers in the diet over separating the individuals because social separation can cause stress[26]. Stress would not be beneficial to the health and welfare of these animals and be a potential risk factor of influencing the study. Within Zoological parks and other wildlife institutions fecal markers are mostly used for identifying individual feces for hormonal and nutritional analyses. The usage of these markers is mostly in Carnivora and Primates. Only beads and glitters are reported to be used in color combinations of 4 colors or more[24]. Commercially used green liquid food coloring has successfully been use with cats housed in pairs, other colors were not distinctive enough in the feces[28,29]. Another research investigated the effectiveness of different colors of bakers' paste and glitters in cats and concluded that both were usable for identification. The downside of the bakers' paste was that it became more visible when given in larger amounts and the taste was extremely bitter. Also, and some irregularities were seen within the stool of the male cats given the bakers' paste. The glitters showed to have no effect on the appetite, bodyweight, and gastrointestinal tract of the cats. This research advises the use of the glitter colors red, green, pink, white, and blue at a dosage of 1/8th teaspoon a day[28]. The colors gold and silver should be avoided in combination with other colors, due to the possible loss of color during digestion. Only nontoxic plastic glitters are recommended for usage. Aluminum glitters were not evaluated in this study[28].

In this study non-consumable, non-toxic, decorative glitters were used and showed no worsening in gastro-intestinal signs. Other researches support our findings that nontoxic glitters did not influence the gastrointestinal system of animals [28,30]. Another research on the efficacy of beads as an identification marker, showed radiographic inconsistent passage and pyloric retention of plastic beads in cats, meaning the size of the beads influenced the passage times[30]. Other reports about markers in lion-tailed macaques mention trying food coloring and baker's paste, before settling with nontoxic glitters. Research to the most effective markers investigated in DeBrazza's monkeys resulted in glitters and plastic beads[24]. The results of this study conclude that the consumable glitters are not effective as a marker for identification of fecal samples of the *M.nigra.* The plastic beads used in this study, were only tested in the laboratory, and four out of eight colors faded in the fecal samples within 24 hours. The colors of beads that did not lose their color were the colors: 'white', 'shiny white', 'silver', and 'black'. The similarity of the remaining colors of beads, and the possibility that the beads could influence the gastrointestinal passage times of the individuals, made the choice of non-consumable-nontoxic glitters preferable over beads.

Furthermore, some of the fecal samples had a Waltham-score of 5.0, meaning that the beads could have scattered throughout the complete enclosure. The eventually chosen colors and dosage of non-consumable, nontoxic glitters of the company 'Rainbow Dust' were: ½ teaspoon of 'Lime green', 'Oasis Blue', 'Chery Red', and 'Bubblegum'. A 100% accuracy in fecal identification was achieved while using these four colors. Even though, part of this success should be credited to the training in successfully feeding the correct colors to the intended individuals, it is mostly achieved due to the visibility of the colors and the distinguishment between them. The knowledge of the behavior and social structures of the group and some training were needed before the correct fecal markers were fed to the intended individuals. Due to their social structure, the dominant animals, and the juveniles, sometimes succeeded in eating the markers intended for another individual.

The study population was given cooked white rice prior to the start of this study, so the possible influence of the rice on the microbiome or digestion rate should be further investigated. Since it normally takes up to 6-8 weeks before the influence of a dietary change can be reflected, the influence of rice is unclear. However, the diarrheal signs were already present before the animals were fed rice, so it might have influenced the situation but is therefore left out as a primary causation.
Concludingly, the feeding of the fecal markers should be done by an experienced person (for example the zookeeper), who if familiar with the animals, their behavior and social structure. The usage of non-consumable-nontoxic glitters is preferred over beads, and the colors: red, green, pink, and blue seem quite distinguishable from one another. However, the company 'Rainbow Dust' in the UK has pulled the complete line of non-consumable glitters from the market, so a different brand needs to be examined before usage. Also, even though the study group did not show any worsening in gastro-intestinal signs, the safety of the usage of this product cannot be ensured.

Fecal examination and digestion rate

The study design for the fecal examination was mostly designed by using a veterinary laboratory protocol for fecal examination[31]. The color and possible contaminations of the fecal samples were recorded. A scoring system for the freshness of the fecal sample was fabricated into the categories: 1-5. A 1 meant that the observer had seen the fecal sample being produced by a specific animal and 5 meant that the fecal sample was dry on the outside and mostly on the inside. These observations were recorded to enable the researcher to make considered choices when it comes to what samples to use for the microbiome and digestive enzyme studies. Considering both factors implicate an age-estimation, they serve as a subjective insight. The Waltham-scores were used to score the consistency of the fecal samples. Even though, the 'Waltham Faeces Scoring System' grades the fecal samples in categorical steps of 0.5, most samples were scored in steps of 0.25, making it a more continuous model than a categorical one. Therefore, the Waltham-score has been treated as a continuous factor within the statistics of this study. The question needs to be made if this was preferable over changing the original data into the official categorical structure of the 'Waltham Faeces Scoring System'. Especially, considering the Waltham-scores of the control groups were observed by a different, yet specialized, individual. The study of Cavett et al. (2021) compared the agreements between the frequently used 'Waltham Faecal Scoring System' with the 'Purina Faecal scoring Chart' and concluded that experienced people (in this case veterinarians were compared to lay people) were often in agreement on fecal scores[32]. The Waltham-score of fecal samples from individual No.1 were substantially higher than those of the other 3 individuals. The Mean pH of the fecal samples of individual No.1 was also substantially higher than the Mean pH of the fecal samples of the other individuals. This indicates that the diarrhea symptoms seen within this group, are mostly seen with individual No.1. The comparison between the Waltham-score and the pH was made to determine if the pH can be a prognostic factor for a higher Waltham-score. The pH was significantly higher in the samples with a Waltham-score of 4.0 or higher (P=.026). Indicating that the diarrheal samples have a significantly higher pH compared to healthy fecal samples. Interestingly, fecal samples of a 'normal' consistency, were diluted with distilled water (with a pH of 7,0) before the pH was measured. The mean pH of the other individuals (No.2, No.3, and No.4) was below 7.0, making it plausible that their original pH was even lower and therefore the difference with the Mean pH of individual No.1 probably even higher. In contradiction to the above, within the control samples, the diarrheal samples showed a substantially lower pH when compared to the healthy control samples. Also, these results showed a substantially higher digestion rate 2. However, the sample numbers used within the comparison of the control samples were very low(*N*=9-29).
When comparing the study group samples to the control group samples, the study group showed a

significantly higher Waltham-score. Which was expected due to the larger number of 'healthy' samples compared to 'diarrheal' samples within the control group. However, no significant difference was found between the pH-scores of both groups. If the pH would have been in correlation with the Waltham-score, it was expected to be substantially different as well, which it was not. Possibly this incongruity is explained by the differences in species.
The digestion rate was examined on two different moments. The first digestion rate was scored on sight in the animal enclosure, scored from 1-7: 1 meant a very good digested fecal sample, without undigested bits and pieces and 7 meant that it was poorly digested with a lot of small and larger undigested pieces. The second digestion rate was scored in the laboratory, while the sample was being prepared for the measurement of the pH. The same scoring system was used for both. A comparison was made between the results of 'Digestion rate 1' and 'Digestion rate 2' between the individuals and showed substantial differences. When the digestion rates are compared within the control groups, both digestion rates have large differences when comparing the healthy group samples to the healthy individual samples. This might be a result of different lightning of locations or an intra-observer bias, or due to the low sample size, conclusions are taken carefully and as followed: Digestion rate 2 may give an indicative differentiation of the digestion rate between 'healthy' samples and 'diarrheal' samples. A difference is found within the control groups when comparing the Waltham-scores, pH, and digestion rates 2 in the 'healthy' group-and individual-samples and the individual- and group- samples from enclosures suffering from diarrhea (Table 2). Overall, the fecal pH is higher amongst the healthy animals and the Waltham-scores are lower compared to the animals with diarrheal symptoms.
An influential factor that needs to be taken into consideration before drawing any conclusions from the control group samples is that there is no telling how many animals these samples represent. When 8 samples are collected from one enclosure, it is a possibility that those samples are originated of only 2 animals. It was impossible to use fecal markers within the control groups and the fecal samples could only be collected while the indoor enclosures were cleaned. Thus, making it impossible to know if the four samples collected from one enclosure were originated from the same individual. Control samples were collected from 5 different enclosures and individual and group samples were collected to prevent too many similarities in origin.
Another difference between the control groups and the study group is their diet. Even though the diet of both groups was recorded, and their diet has a lot of similarities. There are also species differences. In the wild rhesus monkeys are mainly vegetarian, as to *M.nigra* being mainly a frugivores. The diet of the *M.mulatta* consists of fruits, leaves, seeds, shoots, bark, fungi, flowers, and small invertebrates. Their natural diet is composed of mostly plants, insects., seeds, and occasional small mammals, and carbohydrates as their major dietary component. The typical daily ration of commercial biscuits for both male and female *M.mulatta* is approximately 2-4% of their body weight. Purina monkey chow is used in multiple primate centers (3.45kcal/g, 12%fat, 18%protein, 4.14% sugar carbohydrate and 65.9% fiber) [26]. Gastrointestinal transit time may vary from 16-27 hours. This depends on the diet, activity, and stress levels of the animal. Providing supplemental food items, is an important form of enclosure enrichment. The well-being of both species of Macaques is positively influenced by mimicking natural foraging behaviors[26,33].

Microscopic examination

Because the chronicity of this problem and the risks of malnutrition, the possibility of malabsorption was investigated by examining the digestion rate of the individuals. The microscopic examination showed a substantial difference in the number of fat found within the fecal samples of the *M.nigra* group, compared to the *M.mulatta* group. However, it is unclear if this was due to the differences in species, diet, or if this difference can be related to the diarrheal symptoms. Microscopic examination of 13 samples is performed to investigate the digestion rate of the *M.nigra* individuals. This number is too low for any statistical significance but does give an indication of any differences between the number of fatty acids, fat and starch found. In the fecal samples of the eldest *M.nigra* individual(No.1), the number of fat found was much lower compared to the other individuals, and less than 15% of the Mean fat found in the fecal samples of the youngest individual. The fecal samples of the youngest *M. nigra* individual showed almost 4 times the amount of Starch when compared to the other individuals, and almost double the amount of fat in its fecal samples. Differences in the microscopic examination of both slides is that of one slide 20 fields are viewed and the other is completely examined (>40 fields). However, the averages per field should stay the same and therefore the results would not be expected to be different if you would examine the same number of fields on both slides. The reasoning behind the higher number of fields on this slide is to catch the muscle fibers if there were any. The muscle fibers expected to be very low to zero within our animals. Concludingly, it is plausible that the diarrheal symptoms are accompanied with a lower number of fats in the fecal samples. This comparison is made between the *M.nigra* group and the *M.mulatta* group and seen between the *M.nigra* individuals.

Digestive enzymes

Since the diet of the *M.nigra* was structured in the beginning of April it was interesting to see if there were any differences seen between May and June, 2018. No significant differences were found within the *M.nigra* individuals. Then a comparison was made between the *M.nigra* group and the *M.mulatta* group. Even though the numbers in these tests are low, an indication is given that the Mean number of proteases in the *M.nigra* group was substantially lower in the month May, compared to the *M.mulatta* group. Possibly, this difference was not seen in June, due to the amount of 5 samples. Another explanation could be the fluctuation in fecal proteolytic enzymes. When examined in cats or dogs, the fecal samples need to be collected on at least three consecutive days and frozen, before examining the proteolytic activity[34]. More samples and on consecutive collection days would be preferred to get a clearer insight in their digestion rates. Also, their complete diet should be calculated and compared to the results of the microscopic examinations. Furthermore, a larger control group should be examined, preferably of the same species, rather of a species with a similar diet and a closer genetic order as to the *M.mulatta.*

Research mostly reports on amylase and lipase references of different macaques measured in venous blood. Rhesus macaque serum amylase was found to be 729 IUL-1, and in peripheral venous blood 385.4IU L-1(+/- 136.1) as to a lipase of 25.8 +/- 13.0 IUL^-1[35]. A higher amylase can be found in juvenile animals.[25] Unfortunately, it is unadvised to withdraw blood of these animals due to the risks and stress paired to caption and sedation of these animals. Also, research implies that these amylase and lipase values are very species dependent [25,26]. Furthermore, the location of measurement, the age of the animal and specific diseases may influence these results, therefore it is important to have a clearer view of the enzyme levels of activity within a plausible control species.

Amylase is produced by the exocrine pancreas and is secreted in the cheek pouch. It is responsible for the active digestion of starch, by reducing complex carbohydrates at α-1,4-linkages into disaccharide sugars, which then can be hydrolyzed in the small intestines[33]. Thus, when too much starch is found within the fecal samples of an individual, it might be interested to know if that

individual also has a low level of amylase. Unfortunately, we were unable to draw this conclusion with individual No.4. Serum amylase can be found in the parotid gland, pancreas, liver, and urine[36]. Fecal proteases include trypsin, chymotrypsin, and carboxypeptidase A and B. The primarily origin is the pancreas[34]. Pancreatic enzymes (amylase and lipase) will be elevated in case of a pancreatic disease. Pancreatic enzymes are elevated in a significant proportion of human patients with inflammatory bowel disease (IBD). And studies have shown that corticosteroid administration may decrease serum amylase activity[19,34]. Measurement of their activity in serum can be helpful in determining the presence of pancreatitis. However, changes in the enzyme activity of amylase and proteases is dependent on the animals diet, environmental factors, possible diseases, and the microbiome[18,19,34].
Concludingly, fecal examination of amylase- and protease activity is an easy and non-invasive way to get an estimation between individuals or groups. However, the protocol used (from the UVDL) is mainly used for the examination of fecal samples from dogs and cats. The UVDL has a database, to compare their samples with. A control database with a large number of fecal examinations of enzyme activity measurements (in mm) is needed, before this can be applicable to this study group.

Infectious agents and Microbiome

A microbiome is the definition of the bacteria, viruses, fungi, archaea, and protists flora of an animal. A microbiome or flora can be found on an animal's skin, or for example in the caecum. The gastrointestinal microbiome helps the animal with recovering nutrients (such as fatty acids) from plants and other substrates[18,37]. When the microbiome is imbalanced it affects the hosts immune system, making it more susceptible for different pathogens or opportunists.
The microbiome is dynamic, perceived to be primarily driven by host phylogeny [37–39]. Other influences on the microbiome are the diet, social interactions, the age of the host, whether an animal was breastfed as an infant, to even rainfal[40–43]. Due to the multifactorial design of the microbiome and the number of samples collected of our study group. All assumptions are made with caution.
The microbiome results found in this research are derived from a population of 4 female *M.nigra.* This sample size is too small to draw any significant conclusions. Unfortunately, it was impossible to receive microbiome results of another populations (*M.nigra* or *M.mulatta)* to serve as a control group. The results of this study indicate that the microbiome of these *M.nigra* consist mostly of bacteria and in smaller proportions of Archaea, Eukaryota, and Fungi. Viruses were not included in this diagnostic search.
The Firmicutes, Bacteriodetes, and Spirochaetes, were presented in the largest percentile within the study group. Other research discovered that the microbiota of *M.fascicularis* and *M.mulatta* was, however fluctuating, mainly dominated by *Clostridium-Eubacterium, Lactobacillus,* and *Bacteroides* groups[38]. A different study discovered that the main phyla in fecal microbiota of the *M.fascicularis* and humans were Bacteroidetes (10.63%) and the Firmicutes (78.1%). The major genera discovered within the Firmicutes phyla of these cynomolgus macaques included: *Lactobacillus*(38.8%)*, Streptococcus*(11.7%)*, Clostridium*(6.6%)*, Enterococcus* (4.7%)*,* and *Ruminococcaceae*(5.7%)[20]. The major genera discovered within the Firmicutes phyla of this study group included: *Ruminococcaceae* (27.4%), *Lachnospiraceae*(24.3%)*, Clostridiaceae*(12.2%)*, Lactobacillaceae*(6.5%)*, Eubacteriaceae*(6.5%). Suggesting that the microbiota of this study group have a similar microbiota to the cynomolgus macaques investigated in the research of Seekatz et al. (2013). Other studies have discovered an increased inscidence of *Campylobacter* spp., *Shigella flexneri, Yersinia enterocolitica,* adenovirus, and *Strongyloides fulleborni* in samples collected from macaques with chronic diarrhea[16]. The *Campylobacter* spp., *Shigella flexneri, Yersinia enterocolitica,* did not appear to be increased within our study group.
In fact, no substantial differences were found between the bacterial phylae of all 12 samples,

indicating that all individuals had a similar microbiome. When comparing the Waltham-scores <3.5 and >3.5, no substantial differences were found on phylae level. When comparing the individual microbiomes of May and June, also no significant differences were found. Indicating that the symptoms of diarrhea do not correlate with an imbalanced microbiome. However, there were substantially differences within the phylae Elusimicrobia and Planctomycetes when comparing the samples of May and June. However, the sample size is way too small to have substantial evidence for a statistical significance.

When examining the samples per individual (attachment 4 of this study), the Firmicutes Bacteriodetes and Spirochaetes are the largest phylae in within each individual throughout both Months, indicating that their microbiomes are quite stable and similar within the group. However, some individuals show substantial changes between their phylae when comparing the months May and June. For example, the Firmicutes within the microbiome of individual No.2 and the Euryarchaeota, Planctomycetes and Lentisphaerae of individual No.3 have shown large changes, while individual No.1 and No.4 show smaller changes within their phylae. The changes seen within individual No.3 are explanatory for the changes seen in the group sample shown in the results section of this study. But there is more to it. These results indicate that the microbiome might be related to the population of animals. This can be originated in their genes, social group, biographical drivers, environment and housing, similarity in sex, whether the animals are captive or wild, diet, or other factors[26]. More research is needed to determine whether the microbiome of this study population is similar to its species, or as similar to the *M.mulatta* and *M.fascicularis,* considering the influential factors.

If the microbiome is imbalanced, the host can be more susceptible to pathogens or opportunistic infections. All laboratory investigations and specific searches within the microbiome results did not indicate that one specific pathogen was the cause of the symptoms seen within our study group. However, the fecal samples examined in the laboratory of the Zoo and the ones send in for microbial research were reflecting a few days of a multifactorial and fluctuating system. Therefore, this study is giving merely a small insight to a multifactorial problem.

Conclusion

In general, there were a lot of circumstances and factors still unclear when the study design was structured. Resulting in the rising of new hypothesis and new sub-studies. This resulted in the conclusion that this study needed to be the first step of many to follow, since the original goal of this research was to investigate the causation of the chronic intermitting diarrhea in *M.nigra* at the Zoo, and to determine the possible multifactorial influences. This aim was still the overall goal, but it became clear from the beginning that this goal needed to be divided in multiple sub-studied to be achieved in the end. Therefore, the focus of this study, was molded to the most efficient way to identify and examine fecal samples. And to get an insight in the microbial influences of this study group. With this, a foundation was built for other studies to examine the main goal with larger populations, and a more structured examination process.

Acknowledgements

I would like to thank Dr. R.J. Corbee, dr. Y.R.A. van Zeeland, and L. Bruins-van Sonsbeek for their efforts, cooperation, the critical reading of the manuscripts, and their feedback. I want to thank C. Schilp and the staff of the Zoo in question, for their cooperation, efforts and for providing feedback. I want to thank BaseClear facility and Dr. D. Duijsings for the helpful discussions and providing of information on the microbiome results. I would like to thank UVDL for the usage of their laboratory, and T. Altena for the laboratory training and assistance. I want to thank BPRC for providing information and control fecal samples. I would like to thank J.C.M. Vernooij for the assistance on statistical analysis.

Attachments

Attachment 1: Fecal sampling, scoring and conservation

Before entering the indoor enclosure of the *M.nigra*, the animals will be observed over a short period of time. This way the observer can document any behavioral signs of the group. This also allows the observer to see if any of the animals defecate at that moment, which allows the observer to collect a fresh sample. After observing (est. 10minutes), the animals will be locked out of the indoor enclosure and the observer will go through the enclosure to give all the samples numbers and start the macroscopical examination of the fecal samples. After the macroscopical examination of the fecal samples, the samples will be collected and conserved in different ways.

Macroscopical examination of the fecal samples.

This macroscopical examination protocol is developed to structure the fecal sampling, -scoring, and -conservation methods of this study. This way the possible risk of interobserver bias is minimalized and the macroscopic examination of the fecal samples are measurable and repeatable.

The fecal markers (non-consumable-nontoxic glitters: 'Lime green', 'Oasis Blue', 'Cherry Red', and 'Bubblegum' of 'Rainbow Dust') are fed to the four individuals at least 22 hours before sampling the feces. The used glitter colors will show the following colors when found in the fecal samples:

- Individual No.1 → 'Lime Green' visible as different colors of green.
- Individual No.2 → 'Oasis Blue' visible as different colors of blue and sometimes as black.
- Individual No.3 → 'Cherry Red' visible as the color red.
- Individual No.4 → 'Bubblegum' visible as the colors pink, orange, yellow, and slightly light green.

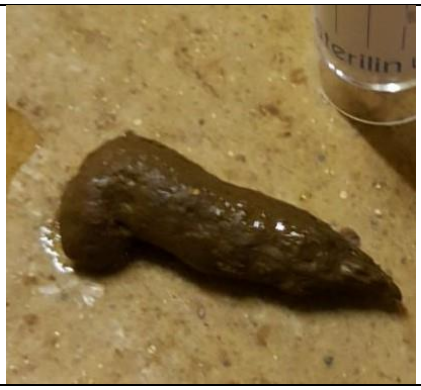
All fecal samples in the indoor enclosure need to be examined (and therefore sampled) to investigate the digestion capacity of the individuals included in this study. An insight into the microbiome of the individuals included in this study is gained by examining the freshest, uncontaminated fecal samples. These samples will serve as representatives for the colonic fecal microbiomes.

<u>Contamination of the sample:</u>

It is therefore important to exclude any fecal samples that are contaminated with urine. If the contamination status is unclear, the sample will be collected and the suspicion of and determination of the possible contamination will be documented (for example: urine, vomit, slime, fresh and old blood, hairs, or others).

Digestion rate of the fecal sample in the indoor enclosure:

The digestion rate is scored in the indoor enclosure and in the laboratory. The scoring system rates from 1-7.

Digestion rate:	Meaning:	Description:	Example picture:
1	Very well digested	No undigested material is visible on or in the fecal sample during the sampling process.	
2	Digested well	The fecal sample looks, well digested. Meaning a minimum of undigested and unrecognizable material is seen on or in the fecal sample during the sampling process.	
3	Properly digested	The fecal sample looks properly digested. Meaning only a few undigested, unrecognized pieces of material are seen on or in the fecal sample during the sampling process.	
4	Properly-moderately digested	The fecal sample looks properly-moderately digested. Meaning only some undigested pieces of material are seen on or in the fecal sample. These undigested pieces mainly consist of small plant-based fibers, seeds, and pieces of eggshells.	

5	Moderately digested	The fecal sample looks moderately digested. Meaning multiple undigested pieces of material are seen on or in the fecal sample. These undigested pieces seen in the fecal sample consist of plant-based fibers, seeds, peels of tomato and bell pepper, and pieces of eggshells.	
6	Moderately-badly digested	The fecal sample looks moderately-badly digested. Meaning a lot of undigested pieces of material are seen on or in the fecal sample. These undigested pieces seen in the fecal sample consist of different sizes of plant-based fibers, seeds, peels of tomato and bell pepper, and pieces of eggshells.	
7	Badly digested	The fecal sample looks badly digested. Meaning most of it consists of undigested pieces of material. These undigested pieces seen in the fecal sample consist of different sizes of plant-based fibers, seeds, peels of tomato and bell pepper, and pieces of eggshells.	

<u>Freshness of the sample:</u>

The freshness of the fecal sample will be rated from 1-5.

Freshness rate:	Description:
1	The observer saw the individual defecate; thus, the sample is not only minutes old when collected(estimation: <1hour), but the origin is also identified
2	The observer did not see the defecation of the sample, but the complete sample is still moist and therefore can only be a few hours old when collected.
3	The surface of the fecal sample looks a little bit dry, but the bottom and inside are still moist when the sample is collected.
4	The surface of the fecal sample is a little bit dried up, but the bottom and most of the inside is still moist when the sample is collected.
5	The complete fecal sample is dried up when the sample is collected.

The color of the fecal sample:
The colors of the collected fecal samples are documented. Fecal sample colors are categorized in: 'green', 'dark green', 'light brown', 'dark brown', 'green-brown', 'orange-brown', 'brown-red', 'yellow-white', and 'others'.

Waltham-Score of the fecal sample:
The Waltham-score will be evaluated according to the 'Waltham Faeces Scoring System'. This scoring system is to be displayed in the indoor enclosure and can be found in attachment 2 of this study ('*Waltham- Faeces Scoring System' Attachment 2 of this study*). The Waltham-score will be documented following the categorical steps of 0.5 from 1 to 5.

Visibility of the glitter color:
Please cross off which does not apply to the fecal sample in question: 'The glitter color is easily visible and distinguishable' or 'the glitter color is not easily distinguishable'.

- The number of glitters found in the fecal sample will be categorically documented from 0-5.
  - '0' meaning no glitters were seen in the fecal sample,
  - '1' meaning a few glitters were seen in the fecal sample,
  - '2' meaning several glitters were seen in the fecal sample,
  - '3' meaning many glitters were seen in the fecal sample,
  - '4' meaning an abundance number of glitters were seen in the fecal sample,
  - '5' meaning an excessive number of glitters were seen in the fecal sample.
- The different colors reflected by the glitters in the fecal sample in the indoor enclosure will be documented. And the suspected original glitter color (or color combinations) within the fecal sample should be documented.

Comments section:
Please use this section to add extra comments to the documented factors above. For example, if any additional comments need to be made concerning the possible contamination of the fecal sample. This section may also include any other possible influencing circumstances, reflecting the behavior of certain animals, or housing/environmental changes that were noticed.

Observer section:
The observer of the fecal samples on a particular day should sign of the document before delivering it to the laboratory. The observer may be contacted if any questions about the fecal sample of the sampling circumstances arise during the rest of the investigation.

Laboratory:
In the laboratory the macroscopic examination of the fecal color, the digestion rate, and the visibility of the glitters will be repeated.

Sampling of the fecal samples

After documenting all the macroscopic features of the fecal samples, the fecal samples are collected. First, the sampling tubes will be numbered representing the fecal sample number. The observer will take a picture of the fecal sample next to the numbered sampling tube, this way the date and sample number becomes traceable.
Then, the date, time of sampling, found glitter color, and the destination of the fecal sample will be written on the label of the sampling tube. The destination depends on the purpose of the

fecal samples (determining the effectiveness of the fecal identification markers / digestion rate / microbiome). The purpose of the fecal samples also influences if all samples are collected, or only the ones with a freshness score 1 or 2. When the purpose of the fecal sampling is to determine the digestion rate or the effectiveness of the markers, all fecal samples are collected. But when the purpose of the fecal sampling is to determine the individual's microbiome, only the uncontaminated samples and the samples with the freshness score 1 or 2 are collected.
The different destinations of fecal samples are:

- Laboratory of the Zoo
- UVDL
- BaseClear

For the investigation of the microbiome and digestion rate of the samples, the amount of fecal sample is important. To examine the microbiome a sample of 3 grams is needed, and a sample of 2 grams is needed for the examination of the digestion rate. Therefore, the samples will be weighed when collected, to make sure there is enough sample before sending it to its destination. An amount of 1 grams is enough for the samples destined to go to the Laboratory of the Zoo.
Example: 'Sample 1(green), date and time of collection, BaseClear'.

At a certain point in this study 1 fecal sample needs to be examined for infectious diseases at the laboratory of the zoo, for the digestion rate at the UVDL, and for the micriobiome at BaseClear. Thus, these samples need to weigh at least 6 grams. However, the fecal samples of the youngest juvenile individual have an average weight lower than 6 grams and will therefore be sampled in this specific situation different from the others. When this specific situation occurs, the samples of the youngest individual will be collected and divided over the sampling tubes as followed:

- 1 sampling tube with 0.5 grams → Laboratory of the zoo
- 1 sampling tube with 2 grams → BaseClear
- 1 sampling tube with 1-1.5 grams → UVDL

<u>Instruction on how to collect samples with different consistencies</u>
When collecting the fecal samples, the inside as well as the outside of the fecal sample will be collected. The lid of the sampling tube has a little spoon attached to it, which allows the observer to collect the fecal sample. When the fecal sample has a high Waltham-score, this spoon is needed to scrape the diarrhea from the surface. Only the inner part of the fecal sample will be collected, and the edges will be left out due to the contamination risk.
When the fecal sample is of normal consistency, the spoon on the lid is used to divide the fecal sample into several pieces without moving it around on the surface. Try to leave the sample in its place while cutting it through, and thereby collecting the upper and bottom surface, as well as the inside of the fecal sample.

In case one fecal sample has multiple destinations (BaseClear, UVDL, and/or the laboratory of the zoo) the multiple pieces will be divided into multiple jars.

Finally, all samples will be brought to the laboratory of the zoo. At the laboratory of the zoo the sampling tubes destined to go to the BaseClear facility will be frozen at -80°C. All other samples will be cooled at 3-6 °C.

Attachment 2: The 'Waltham Faeces Scoring System'

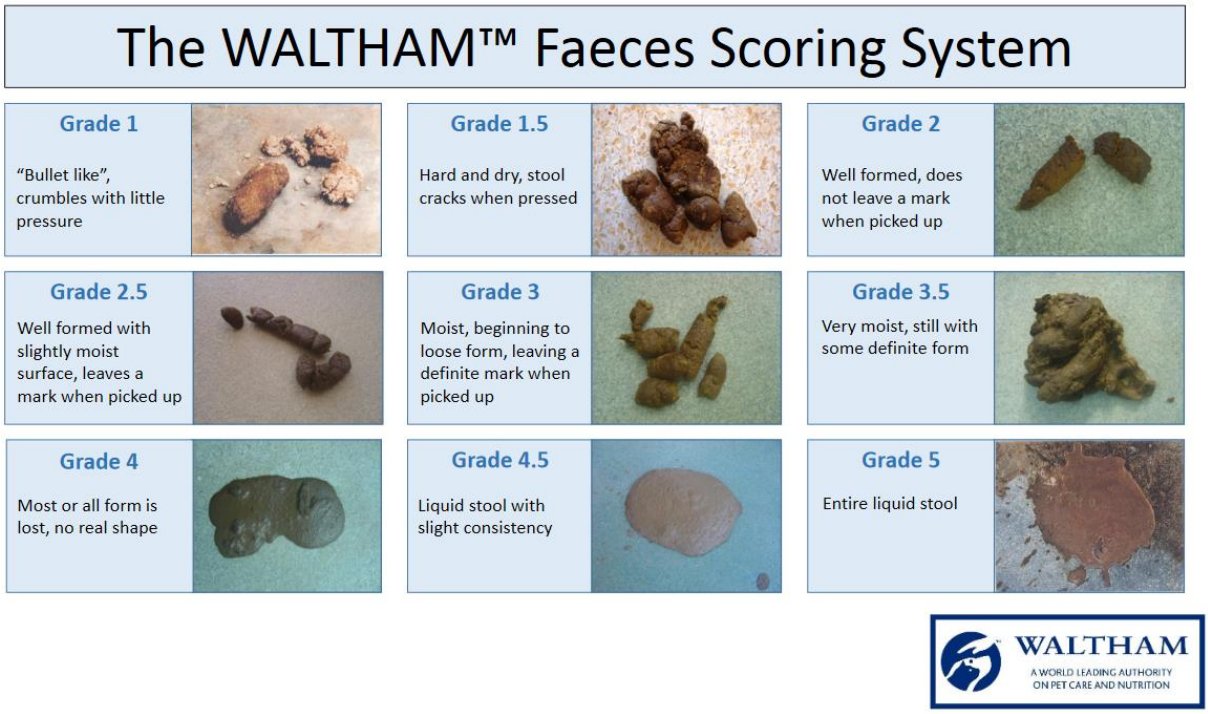


Figure 5: The 'Waltham Faeces Scoring System' developed in 2019 by The Waltham Petcare Science Institute (Cavett et al., 2021)

Attachment 3: 'Protocol faecal identification methods' written by A.Rox (2017) at BPRC

Protocol faecal identification methods

By Astrid Rox (march 2017)

Dosage

- 0.3 – 0.4 ml food colouration
- 2/3 teaspoon feeding glitter of one colour (at least 0.5 a teaspoon, the more the better visible)
- ½ + ½ teaspoon when combining glitters

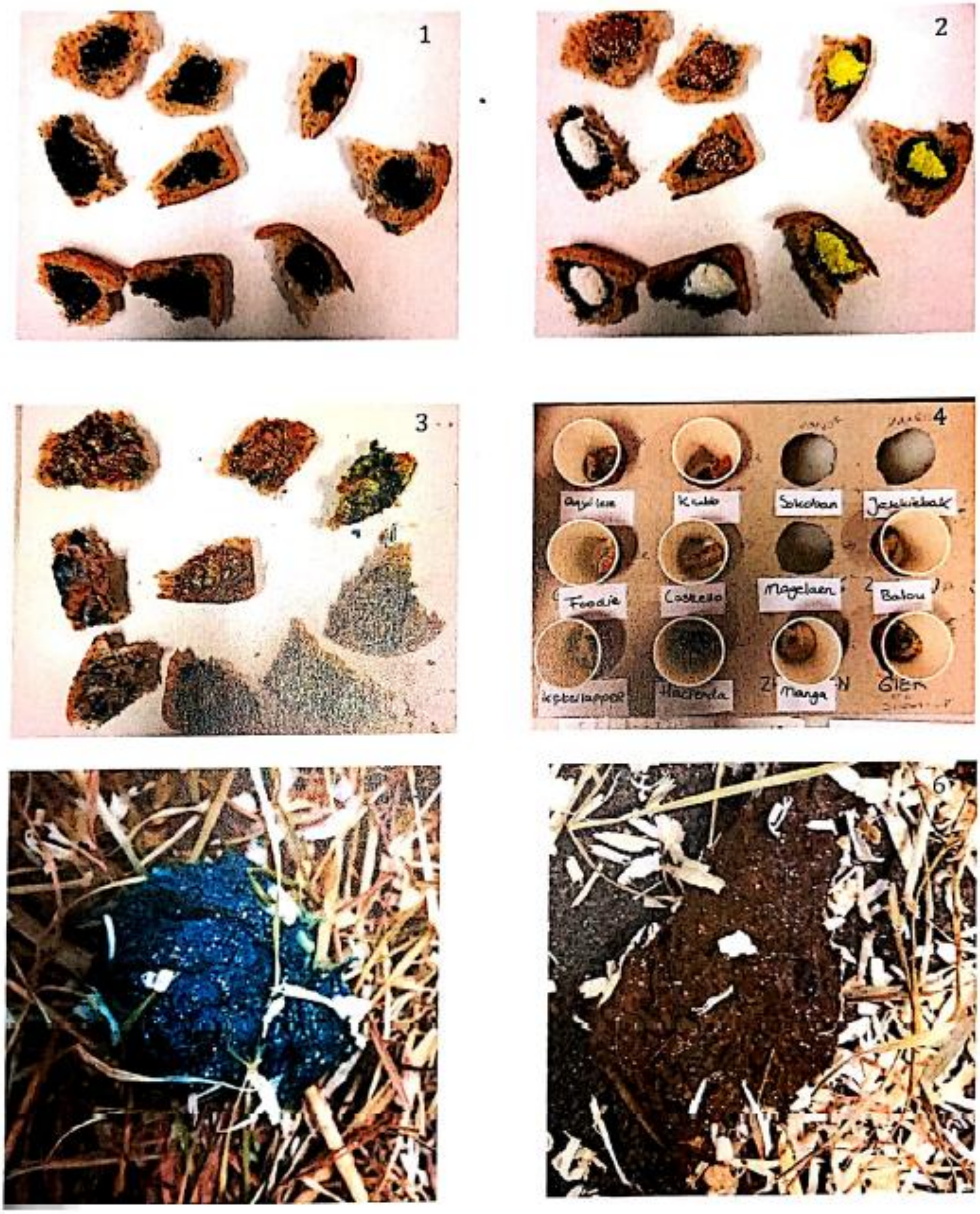
Glitter colours and combinations

- Food colouration: Wilton (USA) – Icing color Royal Blue
- The sparkle range glitter
    - Blue: Jewel Oasis Blue – works fine
    - Light green: Stardust Lime – works fine, difficult to distinguish from yellow
    - Yellow: Stardust Yellow – works fine, difficult to distinguish from light green/gold/silver/bronze
    - Red: Jewel Cherry – works fine
    - Black: Jewel Black Jet – very hard to find back
    - Bronze: Jewel Bronze sand – works fine
    - White: Hologram White – works fine, difficult to distinguish from gold/bronze/silver
    - Lilactic: Glacier lilactic – works fine, difficult to distinguish from gold/bronze/silver/white

- Colour combinations that have been made and worked:
    - Red + Blue
    - Green + Bronze
    - Red + Bronze
    - Blue + Green

Preparing sandwiches

When preparing, take a sandwich and cut it in approximately 9 equal pieces. Take the food colouration and put this on the sandwich with a small syringe (1). Take the glitter and put this on the sandwich (2). Take some peanut butter with a knife and mix this with the glitter on the sandwich (3). Each sandwich will be put in an individual cup to be easily fed to the right animal (4). The remaining part of the sandwich is cut in tiny pieces (1-2 cm big), to be fed to the animals that have already taken their sandwich, or that will not get a sandwich, as a reward (positive reinforcement training).


1
2
3
4
Magelaen
Balou
Manga
6

Schedule

Each morning around 9.30 the animals will be locked out to be able to collect fresh faecal samples in the night enclosure. When you find a sample: mix it and take part of it with you, using clean sticks. Note down the date and colour combinations of the sample. Continue until all combinations have been found. Hereafter, remove the remaining samples from the enclosure, to ensure you have clean samples each day.
Then, the animals are allowed to go in again, and are fed the prepared sandwiches. The glitters and colour will be visible in the faeces the next day, but become better visible after to consecutive days of feeding (5+6).

Feeding

Animals need to learn to individually take a sandwich with glitter, each individual their own sandwich (4). The easiest way to do this is to teach each animal to sit in a particular place, trough positive reinforcement. Only in that place they will get a sandwich, and make sure you are very consequent in this. Try to find a place where animals naturally like to sit, and keep animals that may prevent each other to take a sandwich apart. Animals that may cause difficulty while feeding others (i.e. generally high ranked individuals), should learn to sit in the corridor ("sluis"). Every time someone else is fed and they stayed at their position, they will get a tiny piece of bread as well in the corridor, to teach them to stay in that position (positive reinforcement). When feeding an animal that is close to someone else that may punish them for taking food, or prevent them from taking it, feed them simultaneously.

Attachment 4: Protocol Administering non-consumable-nontoxic glitters

The non-consumable-nontoxic glitters will be administered to the *M.nigra* with 200 grams of cooked white rice, on several occasions between the 1st of April and the 21st of June 2018. The rice was divided into several small balls (3-4cm in diameter) so that these balls would fit through the bars of the indoor enclosure.
It has been discovered that within this specific group it was important to feed each animal its own color of glitters. The animals were given the following colors:

- Individual No.1 → 'Lime Green'
- Individual No.2 → 'Oasis Blue'
- Individual No.3 → 'Cherry Red'
- Individual No.4 → 'Bubblegum'

When one of the individuals is observed to eat from a rice ball intended for another individual, this finding is documented. Thus, it is important that the observer stays and watches the animals until they have eaten their rice balls, before offering them a second rice ball. Keep in mind that these individual shave cheek pouches that they use for momentarily storage of food. Before handling the rice and feeding the animals, make sure that you keep your distance from the enclosure and wear long sleeves and gloves.

Step 1: Flatten per individual two rice balls and divide ½ a teaspoon of glitters (in a specific color) onto the flattened rice balls.

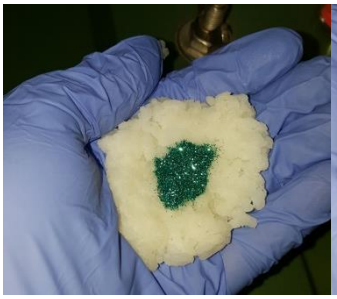


Figure 8: Step 1 of ' Protocol Administering non-consumable-nontoxic glitters'

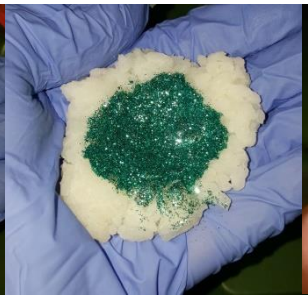


Figure 6: Step 2 of ' Protocol Administering non-consumable-nontoxic glitters'

Figure 7: Step 3 of ' Protocol Administering non-consumable-nontoxic glitters'

Step 2: Carefully spread out the glitters on the surface of the rice ball, leaving the edges clear of any glitters.
Step 3: Fold the flattened rice ball with the glitters inside and roll it back into a ball with no glitters seen on the outside. The diameter of the rice ball should be about 3-4 cm, but for the youngest individual a maximum size of 3cm is used (if necessary, giving 3 rice balls).
Step 4: The most dominant female (in this group individual No. 1) and her young (Individual No.4) need to be fed first and simultaneously, otherwise they might steal from individuals No.2 and No.3. Make sure to keep a distance between the adult and younger individuals in your feeding system. For example, the adult will be given a rice ball through the bars on the right bottom of the door and the younger individual through the left top of the door, this way they are distracted and focused on their own food. The other individuals will be fed their rice balls at the other door of the indoor enclosure, this way the visual contact between the more and lesser dominant animals will be broken, ensuring that they focus on their own food. This enables the observer to keep better track of which individual is finished with their own rice ball.
Step 5: Wait several minutes before giving them a secondary rice ball (if necessary). This because

they tend to put the rice ball into their cheek pouch and running away with it, making it harder to track if they finished their own rice ball.

Step 6: Please write down on the form 'Administering glitters' which color (or color combination) of glitters has been fed to each individual. In case a color combination accidentally occurred, please write down to what extent. For example: 'Individual No. 1 has eaten 2 rice balls containing lime green glitters and 1 ball containing bubblegum glitters. Therefore, individual No. 4 has only eaten 1 rice ball with bubblegum glitters.

Attachment 5: Attachments of the results-section of this study

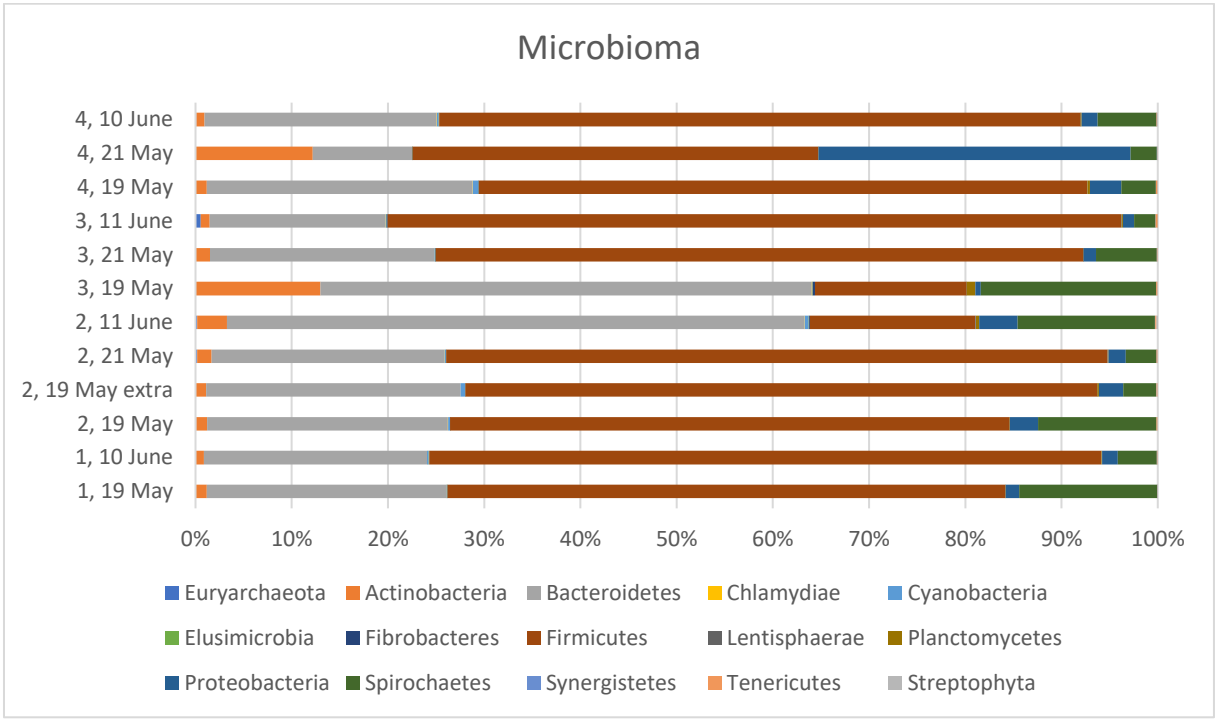


Figure 9: Microbiome percentile overview of the bacterial phylae found in the fecal samples of individual No.1, No.2, No.3, and No.4 of the M.nigra group

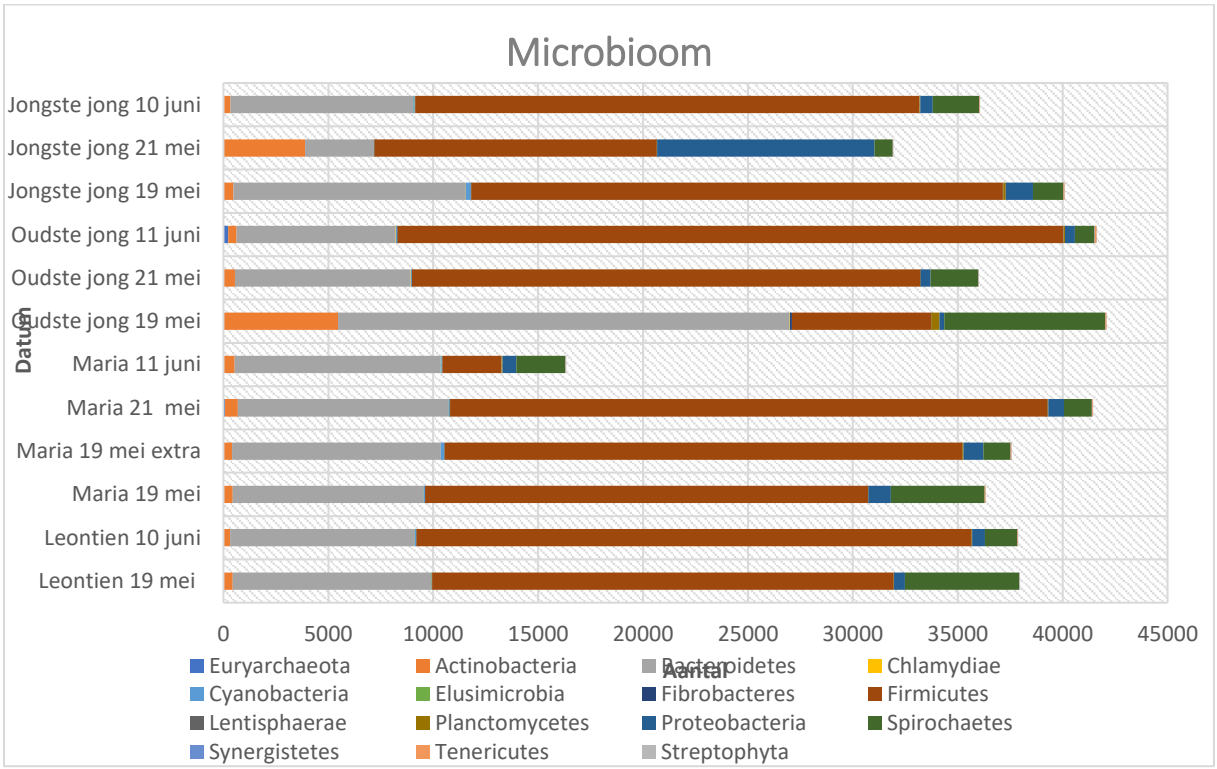


Figure 10: Microbiome overview of the exact numbers of bacterial phylae found in the fecal samples of individual No.1, No.2, No.3, and No.4 of the M.nigra group

Individual No.1 Microbiome results

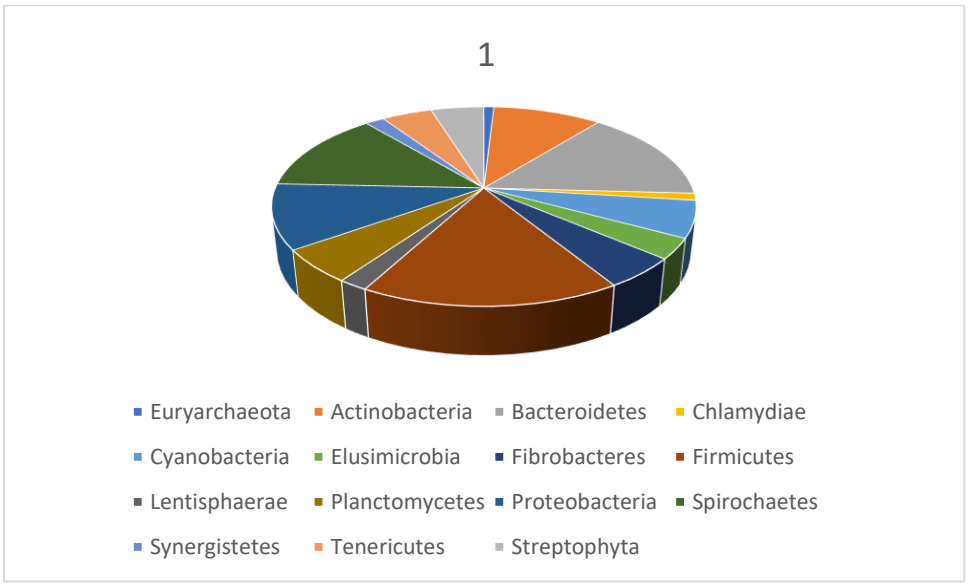


Figure 11: Normalized overview microbiome individual No.1, derived from 2 fecal samples.

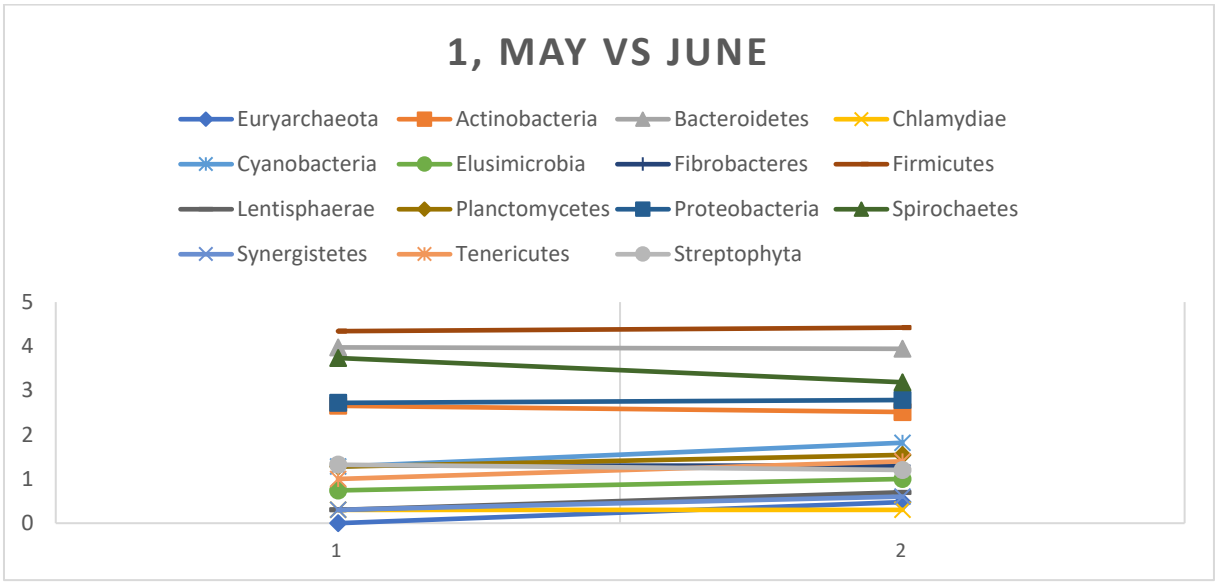


Figure 12: Comparison of bacterial phylae of the microbiome samples in May and June of individual No.1

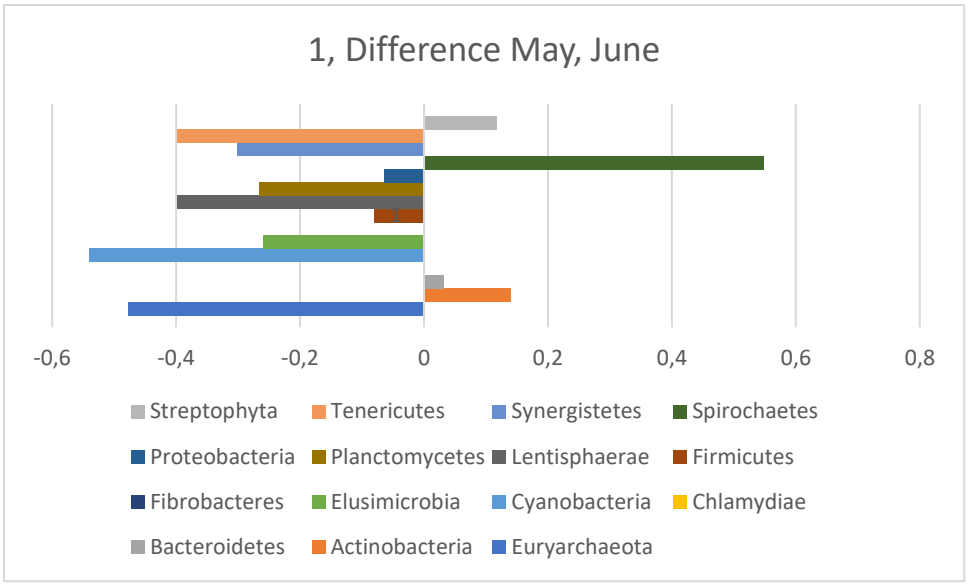


Figure 13: Overview of the comparison of bacterial phylae of the microbiome samples from May and June of individual No. 1

Individual No.2 Microbiome results

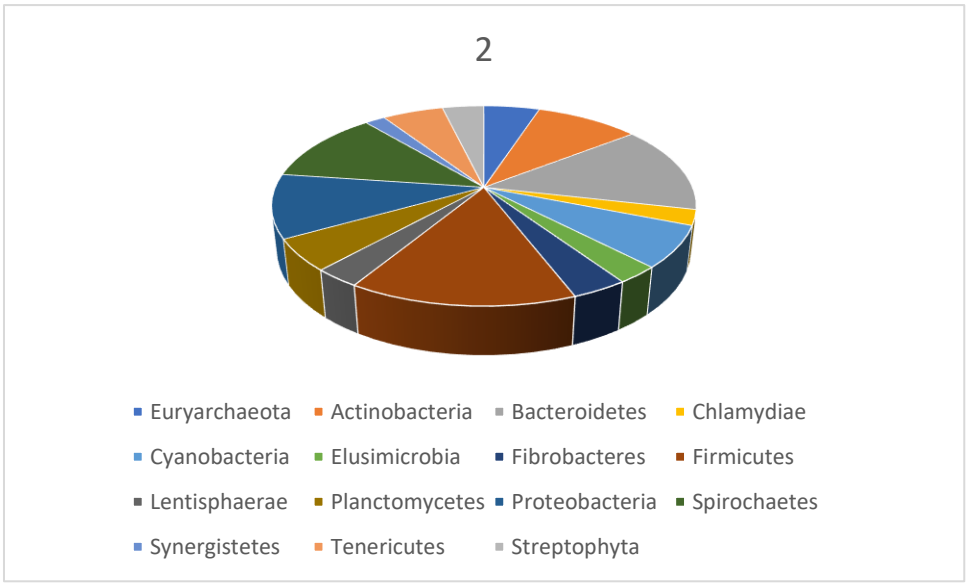


Figure 14: Normalized overview microbiome individual No.2, derived from 4 fecal samples.

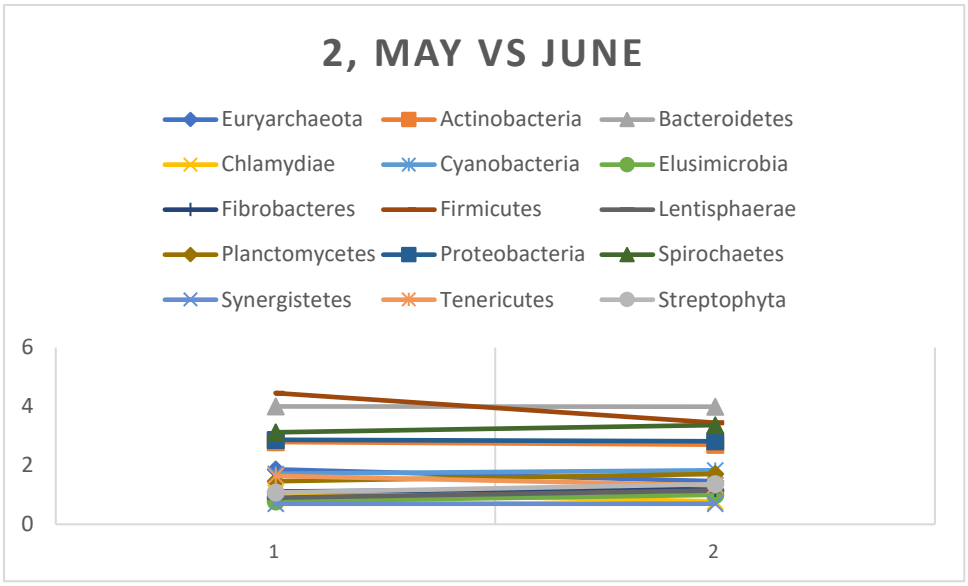


Figure 15: Comparison of bacterial phylae of the microbiome samples in May and June of individual No.2

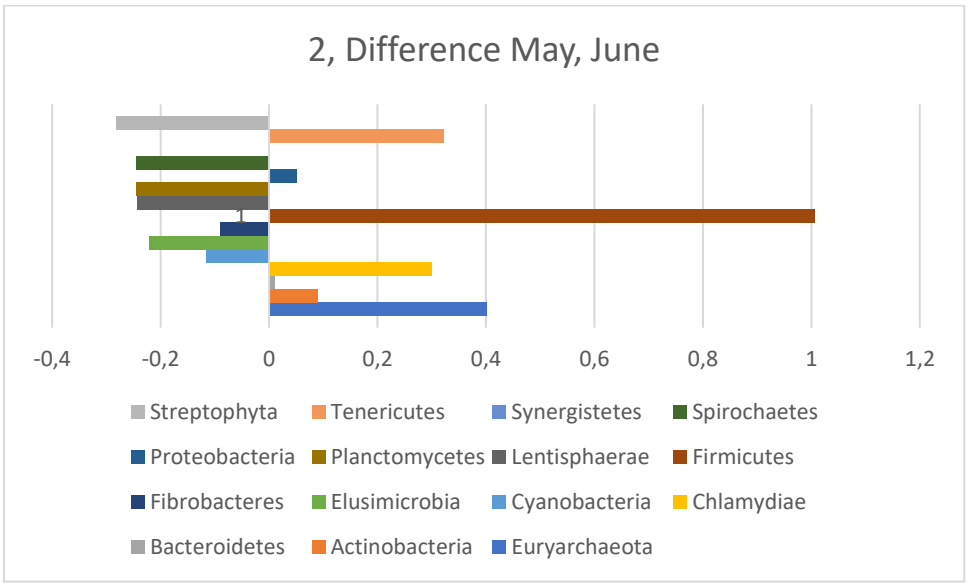


Figure 16: Overview of the comparison of bacterial phylae of the microbiome samples from May and June of individual No. 2

Individual No.3 Microbiome results

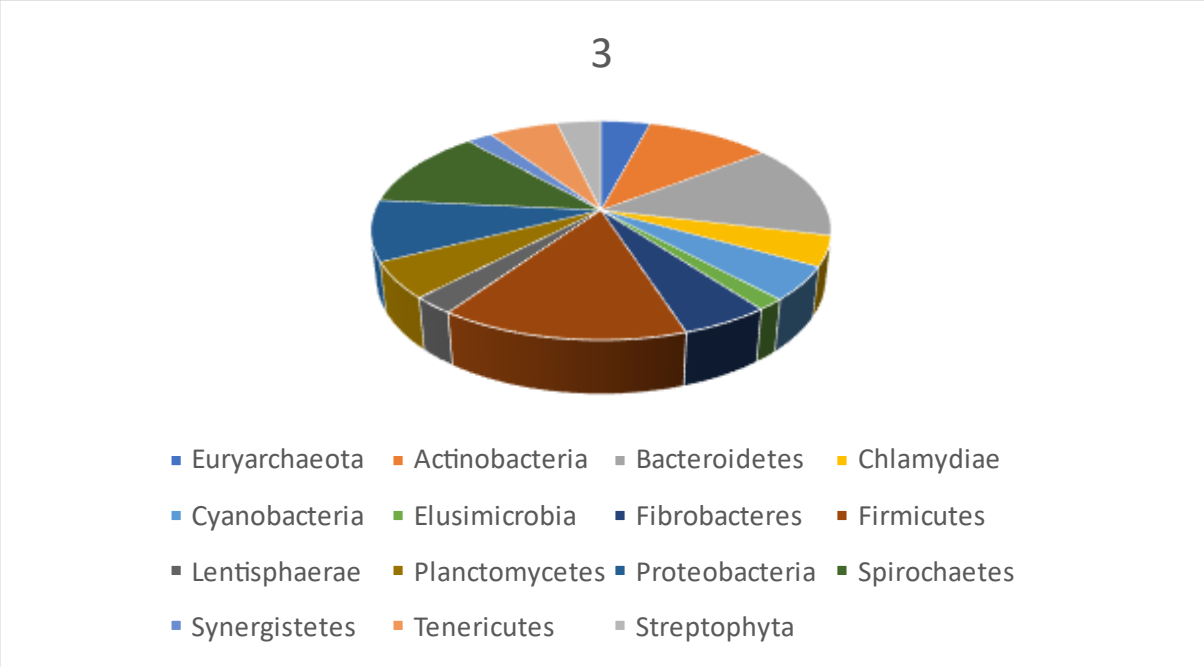


Figure 17: Normalized overview microbiome individual No.3, derived from 3 fecal samples.

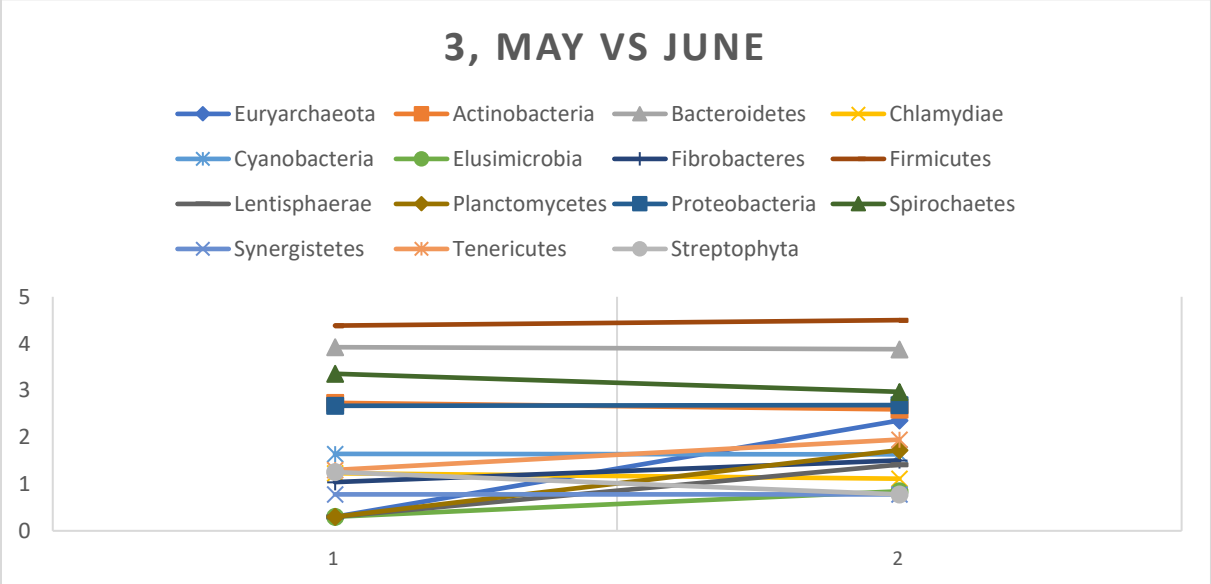


Figure 18: Comparison of bacterial phylae of the microbiome samples in May and June of individual No.3

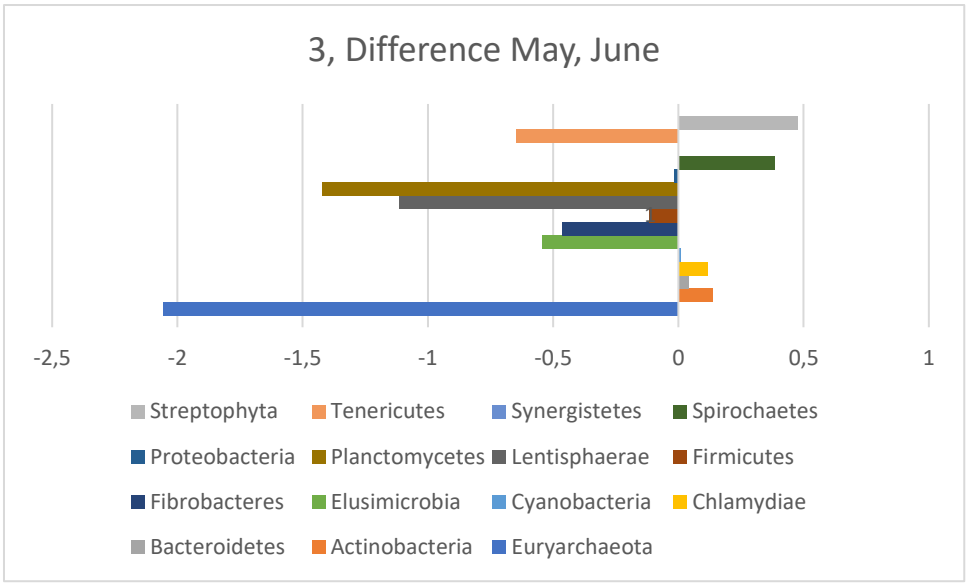


Figure 19: Overview of the comparison of bacterial phylae of the microbiome samples from May and June of individual No. 3

Individual No.4 Microbiome results

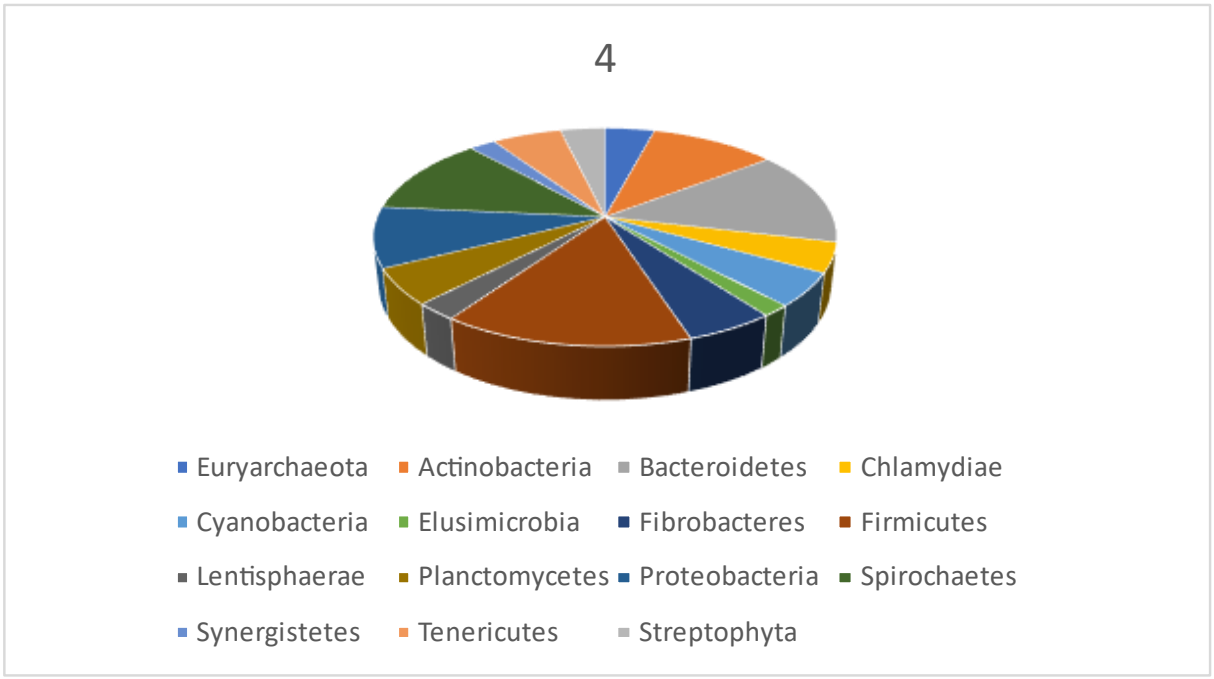


Figure 20: Normalized overview microbiome individual No.4, derived from 3 fecal samples.

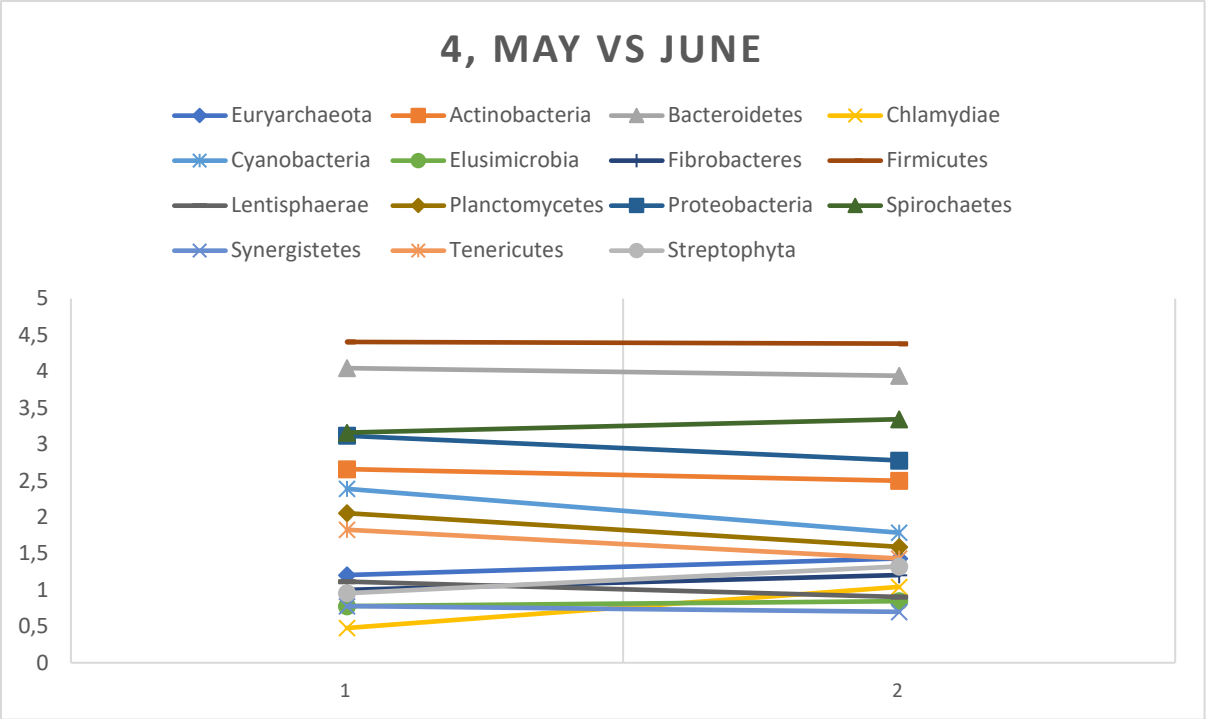


Figure 21: Comparison of bacterial phylae of the microbiome samples in May and June of individual No.4

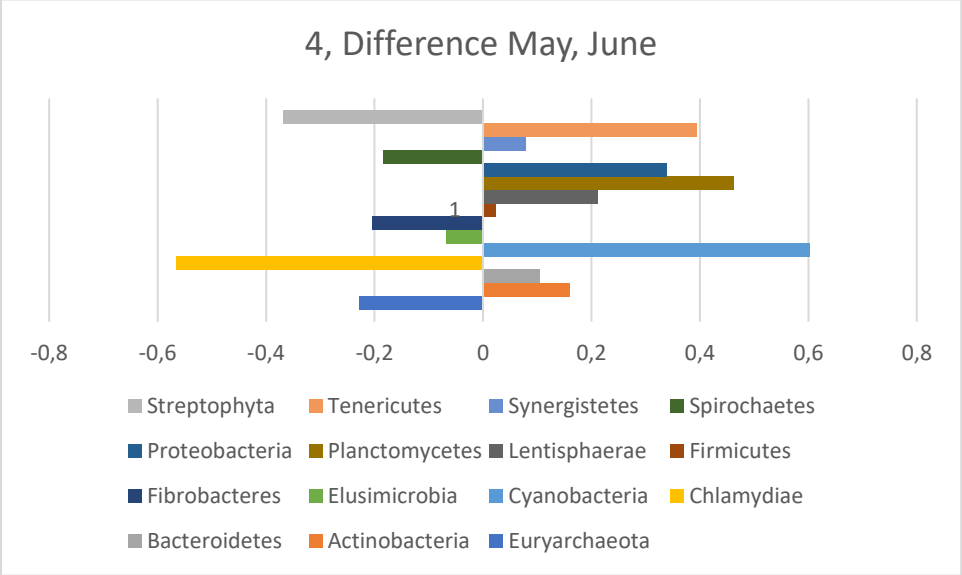


Figure 22: Overview of the comparison of bacterial phylae of the microbiome samples from May and June of individual No. 4

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